A Self-Recording Electronic Osmometer for Quick, Direct Measurement of Colloid Osmotic Pressure in Small Samples[1]

By

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Abstract

Hansen, A. T. *A self-recording electronic osmometer for quick, direct measurement of colloid osmotic pressure in small samples.* Acta physiol. scand. 1961. *53.* 197—213. — A self-recording electronic osmometer for clinico-physiological use is described. It is a further development of an osmometer described earlier by the author. It is based on the passive counter-pressure principle and as such representing a return to the principle that was first applied in osmotic pressure measurements. The general principles for the measuring of osmotic pressure and the special requirements to be fulfilled if quick performance is to be obtained are discussed. The importance of the diffusion phenomena in this context has been emphasized and experimentally elucidated. The sample of plasma or serum which is required, is from 0.02 to 0.1 ml. Ten to 15 measurements can be performed per hour. The confidence limits for one measurement are $\pm$ 0.5 mm Hg. at the 5 % level. Commercially available cellulose membranes are used. No stopcocks are employed. Stainless steel is used for all parts in contact with membrane and sample. The possible usefulness of the osmometer for investigation of the reflection coefficient of permeating particles and for testing membranes is pointed out. Also the possible use in investigations of active trans port problems in connection with organic membranes is hinted at. Finally it is mentioned that the red blood cells under certain circumstances will influence the osmotic pressure.

In spite of the prominent role the colloid osmotic pressure (COP) is supposed to play in the circulation of the blood (STARLING 1896) necessary data for an evaluation of its significance in health and disease are still insufficient. This is to a great extent now as before the result of the lack of entirely satisfactory methods for clinico-physiological studies of the COP.

The *indirect methods* can hardly be considered in exact measurements. Quantitative chemical analysis and ultrafiltration yield results which can only be translated into COP with a wide margin of uncertainty in the individual cases. Freezing point determination and other methods based on colligative properties will fail because of the very low osmolar concentration of the proteins, particularly in the presence of crystalloids, as in the body fluids.

Direct osmometry is the method of choice, but the long time involved in the single measurement, the demand for large samples and the cumbersome procedures in general have restricted the use of the technique in clinical investigation.

By the introduction of electronic pressure transducers into the direct osmometry (TYBJÆRG HANSEN 1950, 1952, MEEHAN *et al.* 1950, PAPPENHEIMER and LIN 1953, ROWE 1954) the possibility of reducing the measuring time as well as the size of the sample was significantly improved. In the following it will be shown that this principle, if thoroughly exploited, permits the design of an osmometer that is rapid, simple and dependable enough for routine use in clinical physiology.

Common principles and problems in direct osmometry with a special view to clinico-physiological demands

The direct measurement of the osmotic pressure of the solution requires a so-called semipermeable membrane. That is a membrane permeable to the solvent but impermeable to the solute. When solvent and solution are placed on either side of the membrane contiguity is obtained through the interstices and osmotic flow begins from the solvent into the solution. This flow will continue as long as there is fluid left in the solvent compartment, at least theoretically. The mechanism by which this flow is generated is not fully understood (MAURO 1957). However it is influenced by hydrostatic pressure as is any other hydraulic flow, and can therefore be slowed, stopped or reversed if a suitable pressure difference is established between the two compartments. The pressure difference, when no net flow takes place in either direction, is per definition the osmotic pressure (π). It is proportional to the osmolar concentration (C) and the absolute temperature (T) with the gas constant (R) as proportionality factor, according to van't Hoff's equation $(\pi = \text{RTC})$. It should be noted that π is independent of the species of the solute molecule.

The zero flow criterion is the basis for all direct osmometry, and this implies that detection of volume flow across the semipermeable membrane is an essential part of the measurement. The volume displacement can be observed directly or

be indicated by its effect on the pressure in one or, more rarely, both of the compartments.

In the so-called static methods (WAGNER 1949), here to be denoted *passive*, the balancing pressure difference is caused by the osmotic flow itself. The equilibrating time is proportional to the relation between the volume displacement and the pressure change it causes.

In the dynamic methods (WAGNER 1949), here to be denoted *active*, the volume displacement across the membrane is only significant as a means to guide the adjustment of an "externally" applied pressure so as to bring about zero flow conditions. For the sake of convenience one of the compartments is as a rule kept at atmospheric pressure, just as the case is in the passive methods.

The active method was introduced (TAMMANN 1892) in order to reduce the extremely long measuring time required by the passive methods. The reason for the slowness of the said methods is the very high flow resistance characteristic of semipermeable membranes. As the necessary liquid displacement across the membrane in principle could approach zero in the active methods the problems of the protracted measuring time seems to be satisfactorily resolved. However the period of fine adjustment in the active method is not much less time-consuming than the last part of the approach to equilibrium pressure in a comparable passive method, *i. e.* with the same sensitivity for detecting volume displacement.

The basic consideration in attempts to reduce the measuring time is, therefore, to increase the sensitivity with which the volume displacement across the membrane can be detected, *i. e.* to increase the modulus of volume elasticity (TYBJÆRG HANSEN 1949).

Electronic methods

It is first of all in this context that the opportunities of electronic methods must be viewed, although they offer other technical advantages, e. g. convenient possibilities of recording. When suitable transducers are employed a very high modulus of volume elasticity can be obtained. The extent to which this favourable property can be exploited depends on the proper design of the rest of the osmometer.

The considerations of *ultra-small volume displacements* do not pose any basically new problems but some of the old ones assume new relative dimensions.

This is true with respect to the *support of the semi-permeable membrane*. A "rigid support" in its practical sense is relative and means that the degree to which the semipermeable membrane yields to pressure is small compared to that of the pressure-sensitive parts, in both cases expressed as volume. The support of the membrane is, therefore, particularly critical when, as here, ultra-small volume displacements are in question. The deleterious effect of an unduly yielding semipermeable membrane cannot be compensated for by any counter-pressure procedures, a fact which has not always been fully realized.

A strictly air-free *filling* of the compartment in which the indicating pressure is to be detected is, of course, necessary in order to sustain the high modulus of volume elasticity of the transducer.

For the same reason the *amount of liquid* held in the pressure chamber of the osmometer should not be too large, as the compressibility of water is not negligible compared to the compliance of the pressure-sensitive part itself (Tybjaerg Hansen 1949).
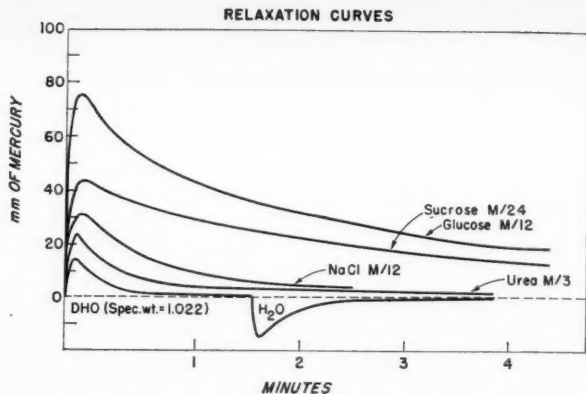
Fig. 1. The curves show the effect of ultimately penetrating substances. The osmotic pressure is related to the osmolar concentration difference at any moment and to the reflection coefficient for the solute in question. The DHO concentration is about 10 M (18 %). The initial reference fluid is in all instances distilled water except in the case denoted H_2O where it is DHO of approximately 10 M concentration. Urea has a remarkably low reflection coefficient.

Whereas for the most part the ultra-small volume displacement leads to exacting demands on the design, it makes it easier in one respect in that it offers the opportunity of reverting to the less complicated passive method.

Even though the preservation of the high volume elasticity coefficient is the primary consideration, attention must be paid to other factors influencing the measuring time, most of them having to do with the properties of the *membrane* itself.

The *water permeability* of the membrane should be as high as possible. Everything else being equal this will mean a membrane with the most numerous and largest possible "pores", i. e. just small enough to withhold the protein molecules. This property is by and large determined by the material of and the manner in which the membrane is made but in addition the *thickness* and the *area* of the membrane are, of course, determinative.

A reduction of the thickness will facilitate the volume flow. So will an enlargement of the area but in both cases it will become harder to secure a rigidly supported, unyielding membrane. Furthermore an enlargement of the area runs counter to the desire of using a small sample, which is important in a number of situations met with in clinical physiology.

Although the reasoning on the whole leads to the recommendation of a relatively thin membrane of small area, the actual dimensions must be chosen as a compromise on the basis of experience.

A matter of particular interest is the *influence of diffusion*, which has been especially clearly brought out during the work with the present apparatus. Generally speaking swift diffusion runs parallel to high permeability of a membrane. The thickness of the membrane plays the same part in connection with diffusion as it does with solvent flow. The area, however, does not influence the time it takes to reach diffusion equilibrium as long as the same relative proportions exist between the area and the volumes in which diffusion takes place.

In order to obtain a quick establishment of the diffusion equilibrium it is favourable to reduce the amount of fluid on both sides of the membrane, preferably to thin layers.

However in order to avoid changes in the salt concentration of the sample it must be large in comparison with the volume of the reference fluid, or rather with that of the effective reference volume. This is defined as that part of the fluid within the solvent compartment in which diffusion equilibrium with the sample has to be obtained and maintained.

The least possible effective reference volume will be that of the membrane or, more precisely, the volume of solvent in the membrane. It is possible to approach this very closely as will be shown.

The reason why a diffusion equilibrium is so important to establish in connection with measurement of COP is that the penetrating molecules and ions exert their osmotic effect according to their concentration at any moment and to their reflexion coefficient (STAVERMANN 1952). Although their effect is equal only to a small fraction of their full osmotic effect as estimated from their osmolar concentration this effect is not at all negligible compared with the COP. Furthermore, and this ought to be particularly stressed, the establishment of diffusion equilibrium cannot be hastened by use of any of the active methods, as diffusion is not much influenced by pressure (MAURO 1957).

In *Fig. 1* is shown how permeating molecules even as small as DHO make themselves osmotically manifest as recorded by the osmometer here to be described.

As long as the diffusion equilibrium is not established the COP measurement cannot be completed. The diffusion conditions are, therefore, a potentially limiting factor in connection with attempts to reduce the measuring time. Besides a reduction of the effective reference volume, as mentioned above, stirring of the sample and selection of the reference fluid before the measurement may be used as a means to enhance the establishment of diffusion equilibrium.

Choice of sample compartment in relation to pressures

The solvent flow is dependent on the pressure difference and not on the absolute pressures in the two compartments. The pressure difference indicating the osmotic pressure may, therefore, be arrived at in several different ways.

From a practical point of view it is highly recommendable to maintain the sample under atmospheric pressure and secure the proper pressure difference by means of a negative (subatmospheric) pressure in the compartment containing the reference fluid. This makes it simple to change a sample, and it prevents contamination of the manometric chamber with protein.

Design and description of the osmometer

The principles here expounded were rather successfully applied in the first version of the osmometer, but it has been possible to exploit the principle more fully in the new one here to be described.

The transducer itself (TYBJÆRG HANSEN 1949) is only slightly modified. The volume of the pressure chamber is reduced to 0.5 ml. The modulus of volume elasticity is slightly higher than the 4×10^9 dynes $\times$ cm.$^{-5}$ of the old one.

The electronic part of the apparatus[1] represents an improvement in performance but there are no principal changes. Only the oscillator and converter but not the amplifier are used. The output from the converter is fed into a

[1] Universal Indicator, DISA ELEKTRONIK, Copenhagen, Denmark.
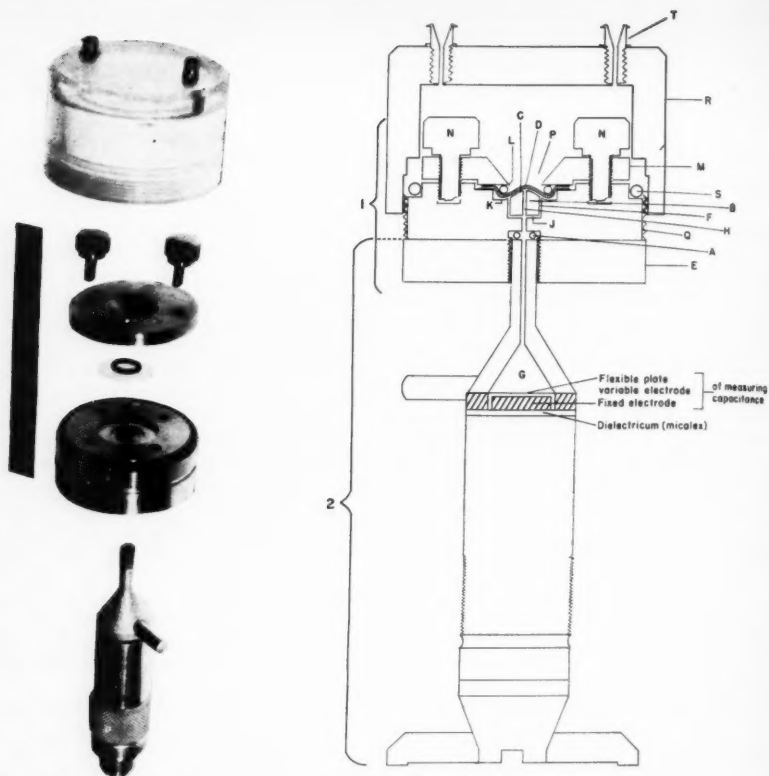
Fig. 2. Axial section of the osmometer and exploded view. Explanation in text.

standard Varian Recorder[1]. The modifications of the transducer and the electronic equipment will be described elsewhere in another context.

The first version of the osmometer was designed as an active method in accordance with the afore-mentioned principles. However as it became evident that it would be possible to achieve equilibrium within a very short time even with a passive method all attention was given to develop the osmometer accordingly so as to avoid the troublesome counter-pressure procedures of the active methods. This decision was further encouraged by the circumstance that the reduction of equilibrating time had reached a degree where active methods had no particular advantages because of the relatively greater influence of the diffusion conditions such as stated above.

[1] Type G-10, full scale deflection 0 to 10 mV in 2.5 seconds. Paper speed 4 inches/hour and 1 inch/minute.

OSMOTIC PRESSURE OF
A 5% SOLUTION OF OX ALBUMIN
AS A FUNCTION OF NaCl CONCENTRATION
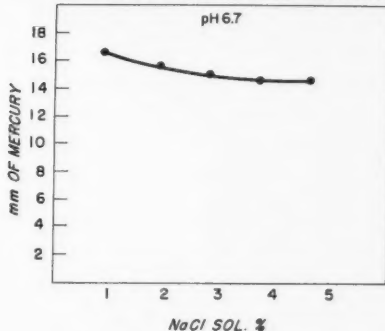
Fig. 3. The effect on the osmotic pressure of varying salt concentrations is a result of the adaptation of the Donnan equilibrium. It appears from the curve that the effect is only slight.

In order to make full use of the high modulus of volume elasticity the stop-cock that was present in the first version was abandoned in order to secure absolute tightness. The result was a noticeable simplification of the osmometer which now consists of two principal parts as shown in *Fig. 2.* and *3:*

The pressure-sensitive transducer. (2)

The clamping unit. (1)

The two parts are assembled by a threaded plug and socket arrangement. The leak-proof sealing is accomplished by an O-ring (A). This arrangement will not only make sure that the system be tight without impairing the modulus of volume elasticity but also that the inner volume and consequently the pressure can be easily changed just by turning the clamping unit slightly with regard to the transducer.

The *clamping unit* (1) is made of stainless steel. The decisive feature is the arrangement for the support of the semipermeable membrane. In contrast with that of the first version the supporting cylinder (B) has a completely smooth surface (C) without grooves of any kind. The membrane (D) rests directly on this surface. The convexity of the surface is increased compared with the first model. The supporting cylinder (B) fits tightly into its socket in the body of the clamping unit (E), leaving a thin, concentric, cylindrical space (F) as main connection to the manometric chamber (G). In the centre of the cylinder is drilled a tiny channel (H), that just permits the insertion of a 27 gauge needle. This arrangement facilitates flushing the space (J) just beneath the supporting cylinder and the concentric space mentioned above. The surface of the supporting cylinder is flush with the immediately surrounding, slightly recessed surface (K) of the body of the clamping unit. Its curvature is smoothly continued so as to form a seat for the membrane (D) and the O-ring (L) so as to bring about a tight sealing in conjunction with the membrane itself. The

curvature is designed so that the O-ring is expanding along the major diameter while being clamped, ascertaining that the membrane obtains a perfect fit to the supporting surface. The clamping disk (M) has two screws (N) which are tightened by hand and two short guiding plugs to facilitate a smooth operation of the clamping procedure. The hole in the clamping disk on the surface facing the semipermeable membrane matches the diameter of the supporting cylinder. Towards the other surface the opening is widening so as to form a conical receptacle (P) with a capacity of 1 ml.

The clamping unit has a thread (Q) on the cylindric surface of the body to provide for a lucite cover (R). The cover has two small openings (T) (female Luer lock adapters) to permit measurements with samples exposed to various gases and gas mixtures. This would also make it possible to operate the instrument as a null indicator, if for any reason that should be desired. Tightness is secured by an O-ring (S).

The way the membrane is supported and clamped creates favorable conditions for the establishment of diffusion equilibrium. The effective reference volume is reduced to that of the membrane itself and the capillary space beneath it. In the cylindrical space (F) and in the tiny channel (H) there is a concentration gradient to the fluid in the manometric chamber (G) but this gradient does not influence the equilibrium in the effective reference volume and is undisturbed by convexion, excepting that brought about by gravitational forces. The design assures that the reference fluid always becomes identical with the ultra-filtrate of the sample in the course of a measurement. No change of fluid of the pressure chamber other than that resulting from the measuring procedures is necessary. The actual effective reference volume is, of course, dependent on the particular membrane used. The membrane which has been found very useful is Schleicher & Schuell, Ultrafine Membrane, Filter Type U. A., Dense[1]. It is 0.1 mm. thick when wet. The area exposed to the sample is $3.5^2\pi = 38$ mm^3. The maximum volume consequently is about 4 mm^3. The volume of the sample is generally 100 mm^3 so that evidently no material change of concentration will ensue as a consequence of diffusion. Even with as small a sample as 20 mm^3, which is the smallest recommended, no effect on plasma COP should be expected because of the difference between the ionic concentrations in the plasma and in 0.9 % saline, assuming no change of pH, an assumption which is justified on the ground of the buffer effect of plasma. This statement is supported by the results of measuring COP in a 5 % ox albumin solution with various concentrations of saline (*Fig. 3*). The slight effect which is apparent requires much larger deviations in salt concentrations than would ensue in the case of plasma versus 0.9 % saline in the above volume relations. It is, therefore, safe to use 0.9 % saline as standard reference fluid. The effect of the salt concentrations is related to the conditions for the Donnan equilibrium (WAGNER 1949).

[1] CARL SCHLEICHER & SCHUELL Co., Keene, New Hampshire.
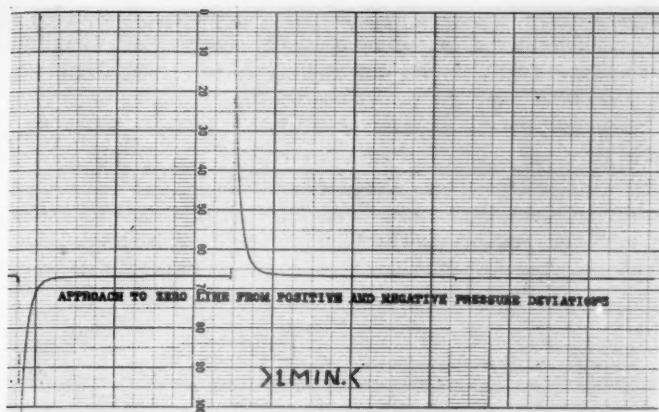
Fig. 4. The figure illustrates the consistency and speed with which the osmometer reverts to zero pressure after deflections in positive and negative direction. Paper speed 15 times that used in routine recordings as displayed in Fig. 5.

Preparation for measurement

Boiling. The transducer and the clamping unit are boiled in distilled water to secure complete filling of all spaces with air-free fluid, and for the same reason all parts are assembled under water.

Connection to the electric aggregate and testing. The osmometer is taken out of the water in such a way that the water remains in the recess at the bottom of which is the seat for the membrane. The connection to the electric apparatus is established and the performance tested. At this stage the osmometer should be checked with respect to pressure sensitivity even though it stays remarkably stable, uninfluenced by any of the procedures involved in its use.

Pressure calibration. The pressure calibration is carried out by means of the lucite cover (R) in connection with a simple mercury or water manometer and a pressure device delivering both negative and positive pressures. The over-all linearity of the transducer is within 2 % from + 300 mm Hg. to — 300 mm Hg., and much better than that in the actual range of measuring from zero to — 50 mm Hg. Whereas a real pressure calibration is hardly necessary more than once for any transducer it is wise to check the sensitivity, *e. g.* every time the membrane is changed and, of course, if the electronic apparatus has been adjusted. One single negative pressure value will suffice for the calibration besides, of course, the zero pressure.

Insertion of the semipermeable membrane is the next step. The membrane is brought to float on the surface of the water left in the recess. The water is sucked away sufficiently much to bring the membrane in touch with the

highest point of the supporting cylinder. The membrane is centered and the O-ring, likewise centered, put on top of it. The clamping disk is put into position on the body of the clamping unit and the screws are tightened until broad contact is made between the two elements of the clamping unit. During the tightening procedure the pressure is increasing in the osmometer but reverts quickly to the same baseline as was observed with the open osmometer. This serves as a check on the fitness of the membrane and shows that the stopcock is unnecessary to check the baseline (*Fig. 4*). The membrane should never be allowed to dry out pending the clamping procedure and it is to be kept covered by *e. g.* physiological saline between measurements. Distilled water is preferred overnight and in other longer periods of idling for the instrument.

Temperature control

The COP is dependent on temperature according to the van't Hoff equation. Ordinary variations in room temperature will, therefore, not disturb the measurement materially. Only if COP is to be measured at other than room temperatures will water bath or similar means to control the temperature be necessary.

However as the baseline for the measurement of COP is temperature dependent too, the problem requires a little more consideration. It is safest to place the apparatus in a water bath, but not necessarily to apply temperature control unless measurement at other than room temperature is required, or the room temperature is more than usually fluctuating. The temperature must then be kept within approximately $\pm\ 0.05°$ C.

A very practical arrangement that takes care of all possibilities is one in which the osmometer is placed in a commercially available 6×6 inches, cylindric stainless steel jar so that the cord to the electric apparatus is entering water tight through a hole made in the bottom. The jar is filled with distilled water to the level of the clamping disk. A stirrer is provided. If other than room temperature is desired water of suitable temperature is run through a coiled-up copper tubing from a temperature-controlled body of water. The arrangement here outlined holds the further advantage that the osmometer may be boiled *in situ*, though partly disassembled, by using an electric dipping heater.

Baseline

The baseline is the level recorded when there is the same osmotic concentration in the effective reference volume as in the receptacle, *i. e.* after diffusion equilibrium is established. As the amount of fluid in the receptacle is the same when the baseline is recorded and when the sample is in the receptacle, there is no correction to be made, neglecting small differences of density. The baseline is independent of the concentration of the fluid in the receptacle. The zero pressure, when the membrane is removed, is identical with the baseline if care is taken to secure the same hydrostatic pressures in both cases.

The baseline is stable within $\pm$ 0.2 mm Hg. for several hours, if the temperature is kept within the limits stated above, and after a couple of hours have been allowed for warming up. Within shorter intervals the variations are even smaller.

If the pressure is raised or lowered by turning the clamping unit the pressure will revert to the baseline very neatly as shown in *Fig. 4*. It will be noted that the approach of the pressure curve to the baseline is symmetrical for positive and negative pressures. It shows that the membrane does not yield more when the pressure is positive inside the manometric chamber than when it is negative, in spite of lacking direct support in the former case. The pressure curves approach zero exponentially in accordance with a Poisseuille type of flow.

Displacement of volume

If the semipermeable membrane were absolutely unyielding and the O-ring between the clamping unit and the transducer completely unpliant the volume change per mm Hg. would be the same for the osmometer as for the transducer, *i. e.* about 3×10^{-4} mm^3. This, of course, is not to be expected.

However a check is easily made by turning the clamping unit a small angle and recording the pressure change. As it can be assumed that only an infinitesimal amount of liquid will have passed the membrane in the short time necessary to turn the clamping unit, the volume/pressure relation will be a valid expression for the elastic properties of the osmometer.

The radius of the connecting tube of the transducer is 3 mm and the pitch of the thread 0.75 mm. A full turn is therefore equivalent to a volume change of

$$3^2 \times \pi \times 0.75 \text{ mm}^3$$

Averaging three experiments it was found that a turn of about $1°$ changed the pressure 133 mm Hg. The actual figures $6/740\ \pi$ were obtained by recording the distance (6 mm) travelled by a 370 mm long extended pointer fixed radially to the clamping unit. The volume change per mm Hg. is consequently

$$\frac{3^2 \times \pi \times 0.75 \times 6}{133 \times 740 \times \pi} \text{ mm}^3 \sim 4 \times 10^{-4} \text{ mm}^3$$

It shows that the efforts to secure a rigidly supported semipermeable membrane have been very successful indeed.

Choice of membrane

The performance of the osmometer as described above relates to the use of the membrane mentioned in connection with the diffusion problem. It is a cellulose membrane and as such tougher and more resistant to both mechanical and chemical injuries than the collodion membranes that were recommended in connection with the first version of the osmometer. It also saves one the
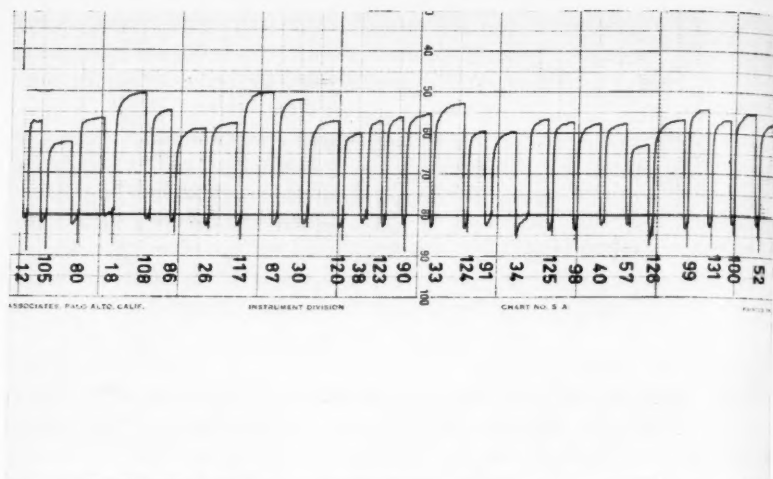
Fig. 5. A series of routine determinations of osmotic pressure of blood serum. The numbers refer to the marking of the samples examined. The zero is at 80 on the vertical axis, the unit of which is 1 mm Hg. The colloid osmotic pressure is read directly in mm Hg. as the difference between 80 and the level of the horisontal part of the upward deflecting. The paper was moving continuously at a speed of 4 inches per hour. One inch is the distance between two heavy vertical lines.

time consuming and often very unrewarding efforts to prepare suitable collodion membranes. However only the further reduction of the volume displacement has made it possible to use a membrane so much thicker. Although most of the membranes from the same batch are usable it pays to pick the most suitable from it. About one-third will function perfectly. It is easily seen on the insertion of a new membrane whether it will be quick enough, as earlier mentioned. Whether it is tight enough is tested by leaving a solution of ox albumin or just one of the samples of plasma in the receptacle and observing that the equilibrium position is kept unchanged for an hour. The receptacle must then be covered by e. g. a small beaker which matches the opening of the receptacle in order to prevent evaporation. The membranes have always complied with this test. Hemoglobin has also been used as test material with the same result. The membranes that have been deemed too slow, however, will give the same result with regard to colloid osmotic pressure but the equilibrium will be reached after a longer period of time, though always within about 15 min. The membrane can be used for months without being taken out of the osmometer and there is no change in its properties. As a membrane apparently can stay in the osmometer for a very long time it is very fortunate that the transducer likewise can be left ready for immediate work over at least as long a period of time with no need for re-calibration or re-setting from day to day.
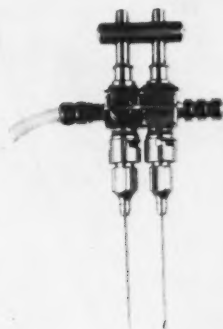
Fig. 6. Combined flushing and suction arrangement. The needle supplied with a polyethylene tube is for suction.

The measurement

In the case of plasma or serum the measurement is performed as follows:

The conical receptacle of which now the semipermeable membrane forms the bottom is flushed with 5 to 10 ml. of physiological saline. The remaining saline is removed by suction and the sample deposited in the receptacle by means of a disposable pipette. The exact size of the sample is, of course, immaterial, only it must cover the whole effective part of the membrane. Generally 0.1 ml. is used as mentioned before. The pressure will immediately start to grow negative in the manometric chamber, as indicated on the recorder. When a plateau is reached after 2 to 4 min the osmotic pressure can be directly read. The next measurement can be made after flushing with saline has taken place. It is not necessary to await a complete return to the baseline, if the temperature control is adequate, and the zero line therefore stable. Ten to 15 measurements can be made per hour (*Fig. 5*). In accordance with the prediction made in the section dealing with diffusion problems flushing with various concentrations of saline does not affect the colloid osmotic pressure of plasma or serum. However the initial pressure course before equilibrium is attained shows variation in accordance with the different concentrations and the osmotic flow due to the diffusing molecules as already demonstrated in Fig. 1. (See the curve for NaCl solution.)

OSMOTIC PRESSURE OF OX ALBUMIN (pH6.7)
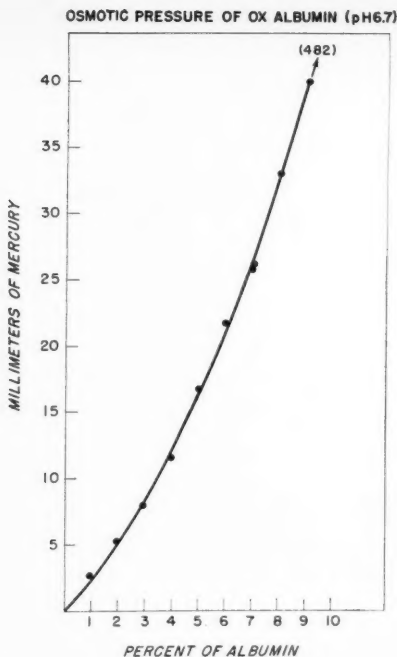
Fig. 7. It is demonstrated here that the osmotic pressure of protein increases out of proportion to the increase of weight/volume concentration. This results (partly) from the fact that the dissolved protein occupies a non-negligible part of the volume of the solution.

Flushing and suction are very conveniently done simultaneously by means of a twin stopcock made from standard units (*Fig. 6*). A 10 ml. syringe is used, filled from a standard saline infusion bottle.

The *confidence limits* of a measurement with all errors compounded is estimated from a series of 10 measurements of 10 consecutive samples from the same serum. Each measurement is carried out exactly as when different sera are used. The limits are $\pm$ 0.5 mm Hg. for the individual measurement at the 5 % level.

Examples of measurements. A few characteristic results of COP measurements are illustrated. In *Fig. 7* is shown the relations of protein concentration and COP over a range of concentrations comparable with concentrations found in sera. The COP is characteristically rising out of proportion to the protein concentration as this latter is growing when the protein concentration is stated on the usual weight-volume basis. The main reason for the lack of proportionality is that the part of the volume of the solution taken up by the protein itself is not negligible as in *e. g.* salt solutions.

In *Fig. 8* the colloid osmotic pressure is compared with protein concentrations in a number of sera. The COP was measured in the same (not normal) sera in which chemical determination of the protein concentration was carried out

OSMOTIC PRESSURE AS A FUNCTION OF
TOTAL SERUM PROTEIN AND SERUM ALBUMIN
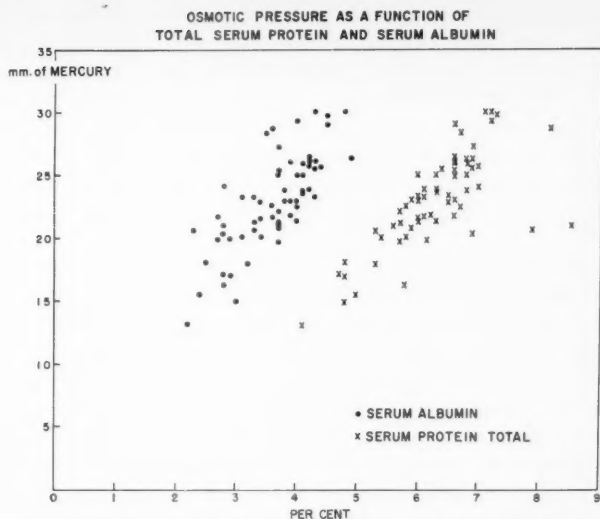
Fig. 8. The osmotic pressure is directly related to the total serum protein values only. The serum albumin values are plotted with the same ordinate as that of the total protein of the sample. The abscissa indicates the actual concentration.

routinely[1]. The over-all agreement between the two groups of results is good, but on the assumption that the measurements are carried out correctly it can also be seen that transformation from one quantity to another can only be made with a wide margin of uncertainty in any single case.

Concluding remarks

As stressed throughout the paper the osmometer has been designed with an emphasis on the measurement of COP in body fluids, the assumption being that the pressure measured is the same as that exerted in the organism. The prerequisite would be that the artificial membrane resembles the capillary wall with respect to molecular size discrimination and that no material concentration of protein is found outside the wall. Although none of these prerequisites are entirely fulfilled the over-all picture is probably not far from being correct (STARLING 1896, KROGH 1929, LANDIS 1927, DRINKER 1937, PAPPENHEIMER *et al.* 1948).

Quite independent of this question the COP measured has, of course, its physical significance and the osmometer may be useful for the measurement of osmotic pressure also in other fields and in other ranges of molecular size if suitable membranes are used. A possible use will be to follow processes in which

[1] For making the samples available I wish to thank ROY W. BONSNES, PH. D., Chief of Chemistry Laboratory, New York Hospital, N.Y.C.

the molecular weight is changed by polymerization or disintegration as a consequence of enzymatic activity.

As shown in Fig. 1 the difference in molecular size is demonstrated by the transient osmotic effect of penetrating particles. It is reasonable to assume that the osmometer could prove useful in a systematic study of reflection coefficients and their relation to total osmotic pressure (STAVERMANN 1952). In this connection it should be mentioned that the author did not observe any abnormal osmosis with various concentrations of urea such as reported in the literature (Grim, 1953).

Instead of using the apparatus for investigating the characteristics of solutions it could be applied to the examination of the properties of membranes. The opportunities for estimating the flow resistance for water is already implicit in the preparation for the ordinary use of the apparatus (see page 10), but could be extended to the testing of other membranes intended for other use.

Attempts have already been made to examine the activity of organic membranes (frog skin) in the apparatus and it is possible that certain aspects of the active and passive transport problems could be elucidated by this technique.

Finally it should be mentioned that the measurement of osmotic pressure of whole blood which was originally anticipated has proved impractical if not impossible. Instead the experiments carried out in this connection have led to interesting observations of the osmotic effect of the erythrocytes themselves under various conditions. Results and their possible implications in the living organism have been reported elsewhere (TYBJAERG HANSEN 1961).

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Attempt to Demonstrate Large Arteriovenous Shunts in Skeletal Muscle during Stimulation of Sympathetic Vasodilator Nerves

By

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Abstract

PIIPER, J. and S. ROSELL, *Attempt to demonstrate large arteriovenous shunts in skeletal muscle during stimulation of sympathetic vasodilator nerves.* Acta physiol. scand. 1961. *53.* 214—217. — The venous recovery of intra-arterially injected wax spheres of different diameters (average sizes 20μ, 30μ and 40μ respectively) was determined in skinned hind leg preparations from cats. Passage of spheres into the venous outflow would indicate perfusion of large arteriovenous shunts. The results provide no support for the assumption that vasodilator nerve activity opens large arteriovenous shunts of any importance from the quantitative point of view.

HYMAN *et al.* (1959) concluded that vasoconstrictor and vasodilator nerves to the skeletal muscle vessels may innervate different types of blood vessels and that the vasodilator effect is primarily on non-nutritional vessels. In order to shed further light on this question, it has been sought to establish whether large-caliber arteriovenous shunts are opened during activity of the sympathetic vasodilator nerves. For this purpose wax spheres of three different average diameters (20μ, 30μ, and 40μ) were injected intra-arterially. The venous recovery of these spheres during vasodilator nerve activity or under resting conditions was determined. It was assumed that the spheres would not pass through true capillaries. Thus, recovery of spheres in the venous outflow would indicate the existence of perfused arteriovenous shunts of sufficient caliber to allow the passage of the spheres.

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214

Table I. *Passage of intra-arterially injected microspheres through the vascular bed of the hind limb muscles of the cat under control conditions and during vasodilator nerve stimulaton*

Exp. no.	Stimulation = S Control = C	Blood flow		Number of spheres injected			Per cent spheres recovered		
		ml/min	%[1]	20 μ	30 μ	40 μ	20 μ	30 μ	40 μ
1	S	12	100	460	405	420	5	3	2
	S	8	100	»	»	»	4	1	1
	C	4	0	»	»	»	2	1	1
2	S	8	80	230	200	210	2	1	1
	C	7.5	60	»	»	»	0	1	1
	S	8	100	»	»	»	1	0	0
3	S	12	50	290	310	360	2	1	0
	C	6.5	0	»	»	»	1	0	0
	S	8	30	»	»	»	3	1	0
4	S	6.5	90	290	310	360	2	1	0
	C	3.5	0	»	»	»	1	0	0
	S	5	65	»	»	»	1	0	0
5	S	11	110	290	310	360	2	0	0
6	C	2.5	0	205	210	230	0	0	0
7	C	7	0	250	210	230	0	0	0

[1] Per cent above resting level, immediately before injection of spheres.

Methods

The experiments were performed on seven cats weighing 3.5 to 4.5 kg. To eliminate the effects of vasoconstrictor nerve activity, reserpine (5 mg/kg) was given subcutaneously 20—24 hours before the acute experiments (ROSELL and ROSÉN 1961). The cats were anesthetized with intravenous urethane (200—400 mg/kg). To prevent clotting, heparin (25 mg/kg) was given i.v. The trachea was cannulated. The arterial blood pressure was recorded in a common carotid by a Statham pressure transducer (P 23 AA). Blood flow was measured in a hind leg by cannulating the femoral artery and directing the blood through a silicone-filled drop chamber operating an ordinate writer (LINDGREN 1958). The blood re-entered the leg via the cannulated distal stump of the popliteal artery. In order to measure only muscle blood flow, the leg was skinned and a tight ligature around the ankle isolated the paw from the circulation. For determination of the number of spheres in the total venous outflow from the muscle region, the distal part of the leg was isolated from the proximal part by mass ligatures just proximal to the knee joint. The only structures left intact were the femur, the nervous supply and the popliteal artery and vein. To maintain warmth and moistness the skin was replaced around the muscle.

The sympathetic chain, isolated and transected via the anterior approach, was stimulated in the distal part with a bipolar silver electrode at the level of L_4—L_5. Supramaximal stimulation produced by a Grass Model S4 stimulator was used throughout.

The recording instrument was a Grass Polygraph.

Injection of Spheres

The spheres were suspended in 2 ml blood from the experimental animal and injected into a closed bypass inserted between the femoral artery and the drop chamber. By shifting the arterial blood flow from the main pathway to the bypass the spheres were injected into the hind leg. The venous blood was collected for a two-minute period, beginning with the injection, by means of a T-cannula inserted in the femoral vein. The number of spheres in the venous outflow was determined by counting them under the microscope (for details see PIIPER and SCHOEDEL 1954).

Results

The results are summarized in Table I. During stimulation of the sympathetic chain the average increase in blood flow was 80 per cent (range 30—110 per cent). The average recovery of injected spheres was 3 per cent of 20μ, 1 per cent of 30μ, and 0 per cent of 40μ spheres. The recovery under control conditions did not differ significantly from that during vasodilator nerve activity. The recovery of spheres was in all cases low.

Discussion

The microsphere injection method has been appraised by PIIPER and SCHOEDEL (1954) and PIIPER (1957). Recovery of intra-arterially injected spheres in the venous outflow is thought to demonstrate the existence of perfused arteriovenous communications of larger caliber than the diameter of the spheres. However, the method provides no information on the morphologic or functional characteristics of such vessels, *i. e.*, no evidence as to whether they are thin-walled permeable capillaries, or thick-walled impermeable arteriovenous shunts. In most cases studied, the passage of 30μ and 40μ spheres appeared to indicate the presence of shunt flow, whereas in some instances the 20μ spheres were apparently able to pass through capillary vessels (PIIPER 1957). Since in the present study the recovery fraction was very small for all spheres, the question as to the type of vessels through which the different spheres passed is immaterial. The very low recovery fraction of the spheres is assumed to indicate the absence of significant perfusion of large-caliber arteriovenous communications both under control conditions and during vasodilator stimulation.

DIETER (1954), using the same microsphere injection method in experiments on the isolated gastrocnemius muscle of the dog, found a considerably higher recovery fraction of intra-arterially injected microspheres: 17.5 per cent of 20μ, 4.3 per cent of 30μ, and 1.2 per cent of 40μ spheres. He was unable to determine whether the relatively high recovery fraction of 20μ spheres was attributable to perfused arteriovenous anastomoses of relatively small caliber, to large capillaries, or to great distensibility of capillaries in the relevant preparation. The quantitative discrepancy between our results and those of DIETER might be due to species differences.

The results of Hyman *et al.* (1959) indicated that the increased blood flow elicited by stimulation of the vasodilator pathway might pass through non-nutritional vessels, since the tissue clearance of radioactive iodide remained unchanged. Our findings do not preclude the possibility that the increased blood flow traversed non-nutritional arteriovenous channels or "functional shunts"; they merely suggest that the caliber of such shunts was smaller than 20μ. Such arteriovenous communications of small diameter and short length, and hence of low resistance, have been reported in skeletal muscle by Zweifach (1937). According to Saunders (1957) arteriovenous shunts of large caliber occur in the vascular bed of human skeletal muscle. The main question is, however, whether such shunts are of any quantitative significance. To judge from our results they are quantitatively negligible, at least under "resting" conditions and during vasodilator nerve activity.

The aim of this study was to ascertain whether the increase in muscular blood flow elicited by stimulation of the vasodilator pathway passes through arterio-venous communications of large caliber (greater than 20μ). Our observations on the skinned hind limb preparations afford no evidence of the opening of large arteriovenous channels during vasodilator nerve stimulation.

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Electrical Signs of the Relation between Caudate Nucleus and Cerebral Cortex in Cats[1]

By

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Abstract

LAURSEN, A. MOSFELDT. *Electrical signs of the relation between caudate nucleus and cerebral cortex in cats.* Acta physiol. scand. 1961. *53.* 218—232. — Cortico-caudate and caudato-cortical relationships were explored in the cat using electrical stimuli and recording with gross and micro-electrodes. The difference in method from previous studies consisted in the use of a 14-lead multilead electrode.

A. *Caudate stimulation and recording from the cortex*

1) A potential elicited in the caudate by stimulation of the caudate was spread to the cortex by volume conduction.

2) Potentials arising in the cortex in response to single shock stimulation of the caudate were not found.

3) Cortical recruiting responses were only elicited from the internal capsule and the thalamus and from a fringe of tissue of the head of the caudate bordering on these structures.

Spread of current from the commonly used stimulating electrodes is thought to explain the contrary results of other authors.

B. *Cortical stimulation and recording from the caudate*

1) No gross response could be elicited in the caudate by stimulation of the cortex.

2) Single units in the caudate responded to single shock stimulation of the ipsilateral motor cortex.

These results confirm cortico-caudate connections, but make even indirect caudato-cortical connections hypothetical.

[1] A preliminary report was presented in Dan. Med. Bull., 1958, 5: 166—167.

There is abundant anatomical evidence of cortico-striate connections (MA-RINESCO 1895, RAMON Y CAJAL 1909—1911, MINKOWSKI 1923, KARIYA 1936, HARISAWA 1936, GLEES 1944). In agreement with this evidence corticostriate connections were found by the method of strychnine neuronography in macaque (DUSSER DE BARENNE and MCCULLOCH 1938, DUSSER DE BARENNE, GAROL and MCCULLOCH 1942) and chimpanzee (GAROL and MCCULLOCH 1944). These findings have been confirmed in the cat (ALBE-FESSARD, OSWALDO-CRUZ and ROCHA-MIRANDA 1960 a) and in addition electrical stimulation of the cortex was found to evoke gross responses (ALBE-FESSARD et al. 1960 a) and responses of single cells (ALBE-FESSARD et al. 1960 b) in the head of the caudate.

On the other hand, with the exception of one report (HARMAN et al. 1954), there is no anatomical evidence of direct caudate-cortical connections (VO-NEIDA 1960) nor were such connections found by strychnine neuronography (DUSSER DE BARENNE and MCCULLOCH 1938). It has been reported that cortical spindle bursts may be elicited by stimulation of the head of the caudate in cat (JUNG and TÖNNIES 1950, SHIMAMOTO and VERZEANO 1954, UMBACH 1959, WIECK et al. 1960). This response was abolished by destruction of the diffuse thalamic projection system (SHIMAMOTO and VERZEANO 1954), a finding consistent with the anatomical evidence (RANSON, RANSON and RANSON 1941, PAPEZ 1942, WOODBURNE, CROSBY and MCCOTTER 1946, GLEES 1945, JOHNSON and CLEMENTE 1959) if the nucleus ventralis anterior and lateralis were included in the lesion. Cortical recruiting responses (DEMPSEY and MORISON 1942) were found to be elicited from the head of the caudate (AJMONE-MARSAN and DILWORTH 1953, STOUPEL and TERZUOLO 1954, WIECK et al. 1960) but in this case too, a direct caudate-cortical pathway seemed to be excluded. When cortical seizure activity was inhibited by single shocks to the caudate (UM-BACH 1959) the effect was thought to be mediated by the intralaminar thalamic nuclei. PURPURA, HAUSEPIAN and GRUNDFEST (1958) and WIECK et al. (1960) reported short latency potentials in pericruciate cortex of cat evoked by stimuli applied to the head of the caudate, indicating direct caudate-cortical pathways.

I am reporting a study in which cortico-caudate and caudate-cortical relationships were explored using electrical stimuli and recording with gross and micro-electrodes. The difference in method from previous studies consisted in the use of a multilead electrode (BUCHTHAL, GULD and ROSENFALCK 1957) allowing well defined changes in the site of recording and of stimulation and evaluation of the extent to which structures other than the caudate were stimulated and might have been responsible for the effects.

Method

Material. Cats weighing from 2 to 4 kg were used. Sixteen were anesthetized with a 1 per cent solution of chloralose injected intraperitoneally in doses averaging 80 mg/kg and 11 were prepared as encéphale isolé under ether anesthesia. Wounds and pressure points were locally anesthetized with tetracaine. Three cats were prepared as cerveau isolé by an intercollicular section and in only one of these could recording be carried out without interference from cerebral edema. Finally, in four cases the animal was cura- rized (LAUDOLISSIN®, ALLEN and HANBURY Ltd.) after wounds and pressure points had been locally anesthetized with tetracaine and the operation performed under ether.

Encéphale isolé. These preparations were studied in the sleeping as well as the aroused state, the sleeping state being characterized by the occurrence of spindles and slow waves in the EEG and arousal by low voltage, fast activity. Preparations which did not show clear behavioral response on pinching the ear were discarded. When cortical re- sponses were to be recorded under conditions of arousal this state was maintained by stimulation of the mesencephalic reticular formation through a concentric electrode using rectangular pulses of 1 msec duration and frequencies of 50 to 200 per second.

Caudate stimulation. The multilead electrode used for stimulation and recording in the area of the caudate nucleus was modified from the multilead electrode described by BUCHTHAL, GULD and ROSENFALCK (1957). For use in the central nervous system it contained 14 platinum leads each 0.1 mm in diameter, distributed along 5 mm of a stainless steel cannula with an external diameter of 1 mm. The distance between the centers of adjacent leads was 0.4 mm. The electrode was stereotactically inserted into the exposed brain and in the different experiments a series of angles and planes of inser- tion was chosen so as to make a thorough exploration of the head of the caudate nucleus. To minimize the damage produced by the electrode and to make it possible to determine its position after the experiment, only one insertion was made in each brain. Stimuli were applied through different pairs of adjacent leads.

For comparison the caudate nucleus and adjacent structures were explored with the following stimulating electrodes: a) Concentric electrode, 0.7 mm in diameter, with a central leading off area of $100 \times 140 \mu$. b) Bipolar electrode, 0.7 mm in diameter, with two $100 \times 140 \mu$ leading off areas. The distance between the centers of the leads was 0.5 mm. c) Unipolar electrode made of a steel needle, 0.8 mm in diameter including two coats of laquer with 0.5 mm bared at the pointed tip.

To reduce artefact the rectangular stimuli of 0.1 to 1 msec duration were applied via a double screened transformer (BUCHTHAL, GULD and ROSENFALCK 1955). Stimulating currents were of the order of 0.2 to 2.5 mA.

Cortical stimulation. Stimuli were applied to the cortex through two steel needle elec- trodes 2 to 4 mm apart.

Recording from the caudate. a) When the multilead electrode was used for recording from the caudate the following arrangements were tested: i) Leading off between pairs of adjacent leads. ii) Leading off between the different leads and the cannula of the multilead electrode. iii) Leading off between one lead of the multilead electrode and a screw inserted in the skull over the frontal sinus. b) Unit potentials were recorded through glass micropipettes filled with 5 M NaCl with impedances of 5 to 10 MΩ. The electrode was advanced by an automatic microelectrode transport device (ANDERSEN and LAURSEN 1959) installed in the roof of a chamber cemented to the skull (DAVIES 1956) and was inserted into the caudate through the exposed intact brain from its dorso- lateral surface.

Recording from the cortex. The following types of recording were used: a) To reveal cal- losal potentials or potentials spread by volume conduction from the depth, recording was carried out from two symmetrical points on both hemispheres through spring loaded

Fig. 1. Thalamically evoked recruiting responses recorded continuously (A) and as superimposed traces (B).

silver ball electrodes resting on the pia. The common indifferent electrode was a screw in the skull over the frontal sinus. Before a response was recorded the indifferent electrode was tested against a silver needle in the temporal muscle to ensure that it did not record responses. b) When studying cortical evoked potentials under conditions of arousal maintained by reticular stimulation it was necessary to eliminate the artefact produced by the reticular stimulation from the record. This was done by leading off between two pial electrodes whose position was shifted until no artefact from reticular stimulation was recorded. c) To study the distribution of evoked changes in the electrocorticogram 6 silver ball electrodes were cemented into holes in the skull in contact with the dura. The indifferent lead was a screw in the skull over the frontal sinus.

When recording was from the pia the dorso-lateral surface of the cortex was exposed bilaterally and covered with a pool of mineral oil. The temperature was measured with a thermistor in contact with the cortex and maintained at 37° C.

Recording apparatus. The electrocorticogram was recorded with 6 channels of an electroencephalograph. Evoked responses recorded with gross electrodes were amplified via a differential amplifier (GULD)[1] with a frequency response of 3 db down at 10,000 and 1 c/sec. The signal from the microelectrode was led through a cathode follower to the same amplifier. The cathode follower added 2 $\mu\mu$F to the capacity of the electrode and input cable. The frequency response employed was 3 db down at 10,000 and 100 cps. The responses from symmetrical points of the two hemispheres were simultaneously displayed on a two-beam oscilloscope. To distinguish evoked gross responses from spontaneous activity 5 to 15 traces were superimposed on the photographic film. The appearance of a recruiting response in this type of recording is shown in Fig. 1 B. Unit responses were recorded using single sweeps triggered from the stimulator.

Determination of electrode positions. To determine the site of the multilead electrode relative to macroscopic structures the heads were perfused with the electrode *in situ*. The perfusing fluid consisted of 5 per cent formalin in Ringer's solution with 5 per cent added colloid (dextran). It had been found that the shrinkage of brain slices in this fixative was about 3 per cent (Knappeis)[2]. After fixation the calvarium was removed and the brain was sliced parallel to the axis of the electrode exposing the leads. The tips of the other stimulating electrodes were marked by the method of MARSHALL (1940) by passing a current from the core of the concentric or from one of the inner leads of the bipolar electrode to a large electrode placed in the rectum. The brains were prepared for histological examination so that the position of each stimulation point could be calculated. The tracks of the microelectrodes were located by histological examination of the brains which had been perfused and fixed with the microelectrode *in situ*. Paraffine sections, cut at 20 μ and stained with thionine were used.

[1] In preparation.
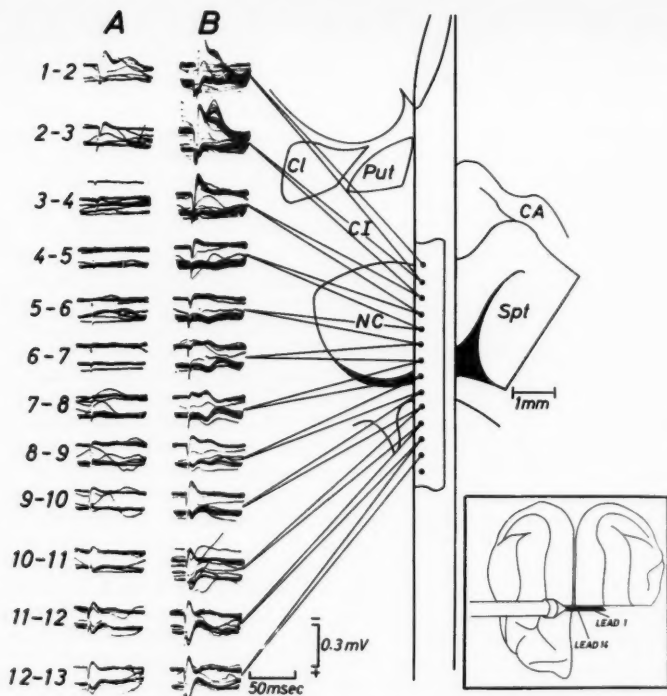
[2] Personal communication.

Fig. 2. Chloralose anesthesia. Responses to stimulation of the caudate and surrounding structures, recorded from symmetrical points of the two anterior sigmoid gyri using a common indifferent electrode. The upper tracing of each pair was recorded ipsilateral, the lower contralateral to the side of stimulation. Fifteen superimposed sweeps. Column A shows responses to 1 msec stimuli slightly above threshold for cortical afferents at 1/sec. Column B shows responses to stimuli at twice the intensity. Weak stimulation between leads 1—2 and 2—3 elicited an early positive-negative response and a later negative recruiting response. Weak stimuli between leads 9—10, 10—11, 11—12 and 12—13 elicited a callosal response. Strong stimuli between leads 5—6, 6—7, 7—8 and 8—9 evoked local caudate responses which were spread by volume conduction (see text) to the cortex and appeared as mirror images in ipsi- and contralateral records. Cortical responses to stimulation in the caudate were otherwise absent. CA Commissura anterior. CI Capsula interna. NC Nucleus caudatus. Pu Putamen. Spt Area septalis.

Results

A. Caudate stimulation and recording from cortex

1. Single shock stimulation

Multilead electrode inserted through the caudate nucleus and adjacent structures. Whether the animal was anesthetized or not single pulses delivered to the caudate did not evoke cortical potentials unless the strength of the stimulus was several times the threshold for cortical responses to stimuli delivered to the internal capsule (Fig. 2). The source of the potential which was evoked from the medial

part of the caudate by pulses exceeding this strength (Fig. 2, column B, leads 5—9) was not situated in the cortex as it was not altered in any respect by local cortical anesthesia or ablation. That it was elicited from the caudate nucleus was demonstrated by the fact that removal of the larger part of that nucleus through a small temporal exposure abolished the potential. The potential was thus spread by volume conduction from the caudate to the cortex. The shape of this potential depended on the site of recording. On the ipsilateral hemisphere (Fig. 2, upper of each record pair) the response had a latency of 10 msec and consisted of a positive deflection followed by a longer lasting negative phase. On the contralateral hemisphere (Fig. 2, lower of each record pair) the shape of the potential was the mirror image of this. The amplitude of the response decreased as the recording sites were moved occipitally.

By stimulation of points adjacent to the caudate cortical potentials were elicited which disappeared after local cortical anesthesia or ablation and must thus be considered to arise in the cortex itself. Stimulation of cortical afferents in the internal capsule evoked a diphasic response with the first phase positive in the ipsilateral sensorimotor cortex (Fig. 2, column A, leads 1—3, column B, leads 1—5). Stimulation of the corpus callosum produced a response (Fig. 2 B, leads 8—13) which could be identified by its bilateral, symmetrical occurrence. Its change from an early positive deflection to a diphasic, positive-negative potential, when the strength of stimulation was increased is in agreement with the description given by CHANG (1953, Fig. 4, records J—R).

Bipolar electrode used for exploration of the caudate nucleus. Stimulating through the bipolar electrode the cortical potential due to volume conduction from the caudate was absent, possibly because the stimulating leads at the tip of the electrode were not oriented properly in relation to the ventricular surface of the caudate.

Concentric electrode used for exploration of the caudate nucleus. Stimulation of the caudate evoked cortical potentials whose latency, shape and amplitude depended on the location of the electrode and the strength of stimulation. No attempt was made to analyse these potentials because the extent to which they were elicited by current spread to other structures was difficult to evaluate.

2. Repetitive stimulation at rates of 1—15/sec

Multilead electrode inserted through the caudate nucleus and adjacent structures

i) With leads in the caudate, recruiting potentials were seen in ipsilateral cortex when the lead pairs were in the head of the caudate immediately adjacent to the internal capsule (Fig. 2, lead pairs 1—2 and 2—3, upper of the record pair). Cortical recruiting responses were absent when stimulation was carried out through lead pairs situated in the rest of the caudate explored (Fig. 2, Fig. 5).

ii) With leads in the thalamus, recruiting responses were evoked in the cortex (Fig. 3, Fig. 4). With lead pairs near the midline (Fig. 3, lead pairs 1—2) the
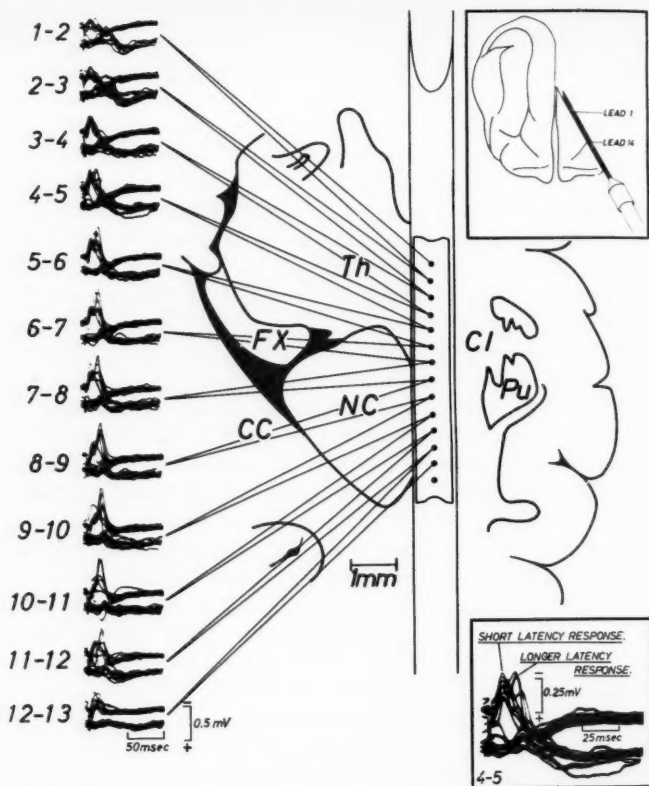
Fig. 3. Encéphale isolé. Recording as in Fig. 1. Repetitive stimulation at 7/sec. The response to stimulation through lead pairs close to the midline (leads 1—2) was bilateral and gradually became ipsilateral only as the stimulus was moved away from the midline in a rostrolateral direction. CC Corpus callosum. CI Capsula interna. Fx Fornix. NC Nucleus caudatus. Pu Putamen. Th Thalamus.

cortical recruiting response was bilateral. As the site of stimulation was moved in a rostrolateral direction the response became more purely ipsilateral (Fig. 3). This characteristic of the thalamic recruiting system was studied by ENO-MOTO (1959) and the relevant literature is reviewed by him. The recruiting system extended continuously from the thalamus through the internal capsule to opposite the rostral pole of the caudate (Fig. 3).

iii) With lead pairs in the internal capsule, recruiting responses were evoked in ipsilateral cortex (Fig. 3).

Fig. 4. Encéphale isolé. Recording between two pial electrodes on the anterior suprasylvian gyrus on the side where the caudate and thalamu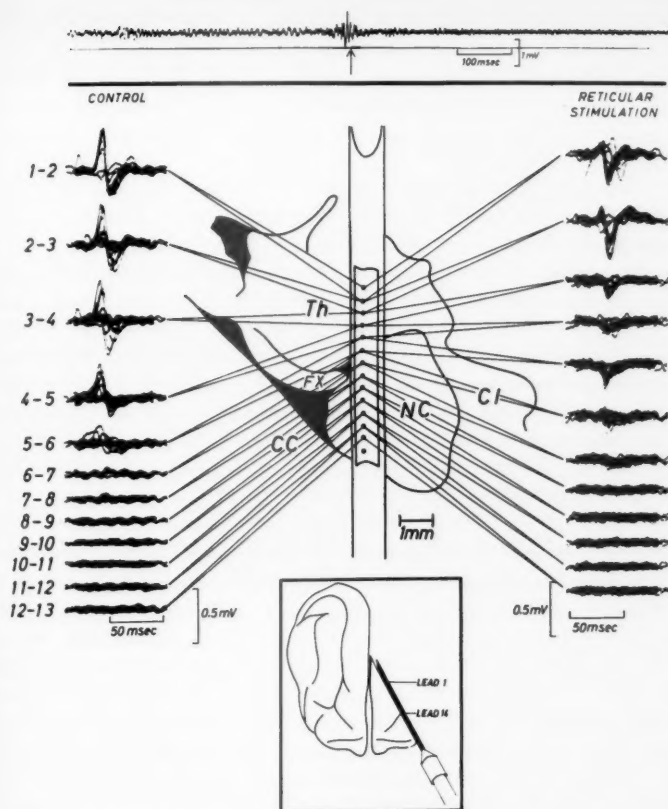s were stimulated at a frequency of 7/sec. Fifteen superimposed sweeps. The column of responses marked control was recorded during the presence of the electrocortical sleep pattern demonstrated in the left half of the top record. The column marked reticular stimulation was recorded during the arousal response illustrated in the right half of the top record. Arousal was maintained by stimulation of the mesencephalic reticular formation through a concentric electrode using 1 msec pulses at a frequency of 215/sec. Arrow marks onset of reticular stimulation. CC Corpus callosum. CI Capsula interna. Fx Fornix. NC Nucleus caudatus. Th Thalamus.

It was found by STOUPEL and TERZUOLO (1954) and confirmed in this study that recruiting responses elicited from rostral areas have short latencies. The experiment illustrated in Fig. 3 showed that the variation in latency in a rostro-caudal direction was not a gradual increase but that two sets of recruiting responses with different latencies were evoked in the cortex. From a rostral area in the internal capsule responses were evoked with stimulus peak times of 7—

10 msec. From a more caudally situated area, overlapping with the former, responses were evoked with stimulus peak times of 16—21 msec.

The optimum stimulus frequency was 7/sec for both groups of recruiting responses. It was lower in anesthetized than in unanesthetized animals (compare Fig. 2 with Figs. 3 and 4) and often decreased as the experimental time increased.

Bipolar electrode. Experiments in which this stimulating electrode was used for exploration of the head of the caudate and adjacent structures gave the same results as those obtained with the multilead electrode.

Concentric electrode. In contrast to the results obtained with the multilead and bipolar electrodes, stimulation through the concentric electrode evoked cortical recruiting responses from the head of the caudate as well as from the internal capsule and thalamus. More spread of current from the concentric electrode was assumed to be the reason for the contrasting results and an attempt was made to measure thresholds for cortical recruiting responses because these were expected to be higher in the caudate than in the internal capsule and thalamus. However, the thresholds varied so much that no significant difference could be ascertained.

B. *Caudate stimulation and recording from cortex combined with reticular stimulation*

Cortical desynchronization evoked by stimulation of the mesencephalic reticular formation is characterized by diffuse cortical facilitation as shown by the increase of the evoked potential recorded in the cortical receiving area when a stimulus is applied to the corresponding thalamic relay nucleus (BREMER and STOUPEL 1959). This is the case in spite of the fact that cortical recruiting responses (MORUZZI and MAGOUN 1949) as well as peripherally evoked sensory responses in the cortex (GAUTHIER, PARMA and ZANCHETTI 1956) are depressed during arousal.

The possibility that cortical responses to caudate stimulation would appear only during arousal was investigated by exploring the head of the caudate with single shocks and slow repetitive stimulation delivered through the multilead electrode, while attempts were made to record potentials from the cortex. Cortical arousal was maintained by stimulation of the mesencephalic reticular formation at 200/sec. No cortical responses were found (Fig. 4, leads 6—13).

Recruiting responses elicited from the thalamus and recorded between two electrodes resting on the pia exhibited changes in shape during arousal in addition to the expected reduction in amplitude (Fig. 4, leads 1—6). No analysis of the mechanism of this phenomenon was made.

C. *Cortical stimulation and recording from the head of the caudate*

1. *Gross response*

The dorsolateral surfaces of the hemispheres were explored with the stimulating electrode using frequencies from single stimuli with 2—3 sec intervals to

200/sec and strengths up to 20 V, while attempts were made to detect an electrical response at the individual leads of the multilead electrode situated in the head of the caudate. The result of the search was negative in contrast with the findings of ALBE-FESSARD et al. (1960 a). The discrepancy may be due to the systematic use by these authors of at least 3 sec intervals between stimuli in animals anesthetized with chloralose and 10 to 20 sec intervals in unanesthetized preparations.

2. Unit responses

It was confirmed that single units in the head of the caudate respond to single shock stimulation (2 to 3 sec intervals) of the ipsilateral motor cortex (ALBE-FESSARD et al. 1960 b) in unanesthetized cats. Stimulating at constant intensities just above those required to produce movement (as determined prior to curarization), one or two spikes occurred, the first with a latency of between 15 and 45 msec. Areas outside the ipsilateral motor cortex were not explored.

ALBE-FESSARD et al. (1960 a) found very short latencies for caudate gross responses elicited from ipsilateral motor cortex and inferred a direct pathway. The latency of unit responses found by these authors (ALBE-FESSARD et al. 1960 b) was of the same order (averaging 27.5 msec) as that found in this study.

Discussion

Cortico-striate connections demonstrated by strychnine neuronography (from areas 4 s, 8, 24 and 2) in macaque (DUSSER DE BARENNE and McCULLOCH 1938, DUSSER DE BARENNE, GAROL and McCULLOCH 1942) and chimpanzee (GAROL and McCULLOCH 1944) have been confirmed in cat. Here they arise from bilateral motor cortex and sensory cortex I and II of both sides (ALBE-FESSARD et al. 1960 a, b), and were confirmed from ipsilateral motor cortex in this study. Direct connections in the form of unmyelinated collaterals from cortico-fugal fibers were found histologically (GLEES 1944) and direct connections were also indicated by the short latency of gross responses evoked in the caudate from the motor cortex (ALBE-FESSARD et al. 1960 a). The long latency of unit responses found by ALBE-FESSARD et al. and in this study has been attributed by her to transmission in the caudate.

On the other hand the existence of direct caudate-cortical connections is very doubtful. Recently VONEIDA (1960) using the Nauta-Gygax silver impregnation technique found no degeneration in the cortex after lesions in the head of the caudate in monkeys and cats. In the study reported here neither responses evoked by single shocks nor recruiting responses (as originally described by MORISON and DEMPSEY (1942)) could be elicited by electrical stimulation confined

to the head of the caudate nucleus. It is true that only sporadic attempts were made to elicit "spindle tripping" (as originally described by DEMPSEY and MORISON (1942)). Failure to find cortical responses to stimulation of the caudate was not due to damage produced in it by the multilead electrode because: i) Cortical responses were obtained from areas outside the caudate where the damage produced by the multilead electrode was the same. ii) The negative results were confirmed using a bipolar stimulating electrode of small diameter and conventional design. Spread of current from the commonly used concentric electrodes were thought to explain the results reported by other authors (AJMONE-MARSAN and DILWORTH 1953, STOUPEL and TERZUOLO 1954, PURPURA, HAUSE-PIAN and GRUNDFEST 1958, WIECK et al. 1960).

Spread of current from the multilead electrode is not less than from a conventional bipolar electrode. The advantage of the multilead electrode is the well-defined mutual localization of the leads which permits comparison of excitability of different locations in the brain without movements of the electrode. Moving the bipolar electrode through the brain introduces a source of error because the soft and sticky brain tissue may move along with the electrode in unpredictable steps.

A reservation must be made. Stimulation of the fringe of tissue of the head of the caudate bordering on the internal capsule and thalamus did elicit cortical recruiting responses. This may indicate current spread within small distances even when stimulation was between small electrodes 0.4 mm apart (multilead and bipolar electrodes). Or it may indicate the presence of unspecific thalamo-cortical fibers in the part of the caudate adjoining the internal capsule. Such fibers have been shown to ascend in the internal capsule in close proximity to the head of the caudate (NASHOLD, HANBERRY and OLSZEWSKI 1955) and the border between internal capsule and caudate is not sharply demarcated.

Some comment on the study by PURPURA, HAUSEPIAN and GRUNDFEST (1958) is necessary because these authors recorded from the medullary pyramids as a measure to detect spread of current to the internal capsule. PURPURA et al. found short latency potentials in the ipsilateral, pericruciate cortex of the unanesthetized cat on single shock stimulation of the head of the caudate. The potentials were initially positive and either polyphasic or diphasic of long duration. Longer latency, long duration responses were occasionally observed in the lateral gyrus.

The pyramidal monitoring used by PURPURA et al. as a precaution against spread of current is insufficient because: a) Spread to the internal capsule would be revealed by pyramidal monitoring only if the threshold of cortical afferents were the same as or higher than that of the efferent fibers. b) In addition to the internal capsule, corpus callosum and thalamus, notably its nucleus ventralis anterior (HANBERRY and JASPER 1953), are areas to which current from an electrode in the caudate may spread, eliciting a variety of simple and mixed cortical responses.

If direct caudate-cortical connections do not exist two questions arise: a) Does the caudate influence cortical activity? And in case it does: b) By which subcortical relay?

a) The experiments reported here showed that neither cortical responses to single shocks nor cortical recruiting responses were in fact evoked from the caudate. LAURSEN (1961) found that electro-cortical arousal could not be elicited from the head of the caudate. It is possible that also cortical burst tripping was in fact elicited from areas outside the caudate. Further evidence for caudate influence on cortical activity was the "suppression" produced in areas L4s and A4s of the monkey by strychninization of the head of the caudate (DUSSER DE BARENNE and McCULLOCH 1938). However, the description of the suppression corresponds in this case to the spreading cortical depression of LEAO (1944). UMBACH (1959) described inhibition of cortical seizure activity elicited by single shocks to the caudate. The results obtained by this author are difficult to interpret because he used stimulating electrodes with up to 3 mm interelectrode distance and stimulating currents from 2.5 to 25 mA (approximately 10 times the currents used in the experiments reported here).

b) Even if caudate influence on the mentioned types of cortical electrical activity is questionable, such influence in a broader sense remains a reasonable hypothesis. The recently demonstrated caudate participation in processes of learning (ROSVOLD and DELGADO 1956, ROSVOLD, MISHKIN and SZWARCBART 1958, BÄTTIG and ROSVOLD 1959, DEAN and DAVIS 1959, THOMPSON 1959) would otherwise be difficult to understand. Recently VONEIDA (1960) showed that caudate outflow is limited to the globus pallidus and the substantia nigra in cat and monkey (macaque). Signals could be relayed from the globus pallidus to the cortex via nucleus ventralis anterior and lateralis of the thalamus (PAPEZ 1942, GLEES 1945, WOODBURNE, CROSBY and McCOTTER 1946, JOHNSON and CLEMENTE 1959, UMBACH 1959). The optic thalamus was a relay for the cortical suppression elicited from the caudate by DUSSER DE BARENNE and McCULLOCH (1938).

In this study a response evoked in the caudate by stimulation of the caudate was recorded from the cortex to which it had spread by volume conduction. Local caudate potentials have been reported previously by UMBACH (1959) and by PURPURA[1].

UMBACH found local spindles preceded by a "Vorwelle" and a "Hauptwelle" in response to single shocks to the caudate but for reasons mentioned above it is difficult to evaluate his results. PURPURA stimulated the exposed ventricular surface of the caudate and evoked a focal, negative potential, similar to the response recorded in this study.

In concluding I wish to point out that the caudate in relation to the recruiting response behaves as the cerebral cortex. Recruiting responses can be evoked in both of these telencephalic areas as well as in the thalamus by stimulation of

[1] Personal communication.

the thalamus (VERZEANO, LINDSLEY and MAGOUN 1953), but recruiting responses cannot be evoked by stimulation confined to the telencephalon. It may be significant in this connection that the morphological substratum for the synapse in the striatum appears to be predominantly represented by a pericellular plexus very similar to the pericellular plexuses of the cerebral cortex, in which terminal boutons, if present, are very few in number (BIELSCHOWSKY 1919, GLEES 1944).

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Caudate Nucleus and Electrocortical Activation in Cats

By

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Abstract

Laursen, A. Mosfeldt. *Caudate nucleus and electrocortical activation in cats*. Acta physiol. scand. 1961. *53*. 233—238. — A 14-lead electrode was used to search for areas in and around the caudate nucleus from which electrocortical arousal could be elicited. Electrocortical arousal was induced from the reticular nucleus of the thalamus and the internal capsule and, probably by current spread, from the fringe of caudate tissue adjacent to the thalamus, but not from other portions of the caudate. The contrary results of other authors were ascribed to spread of current from the commonly used stimulating electrodes. It is concluded that there is no evidence to support the view that the caudate is a part of, or closely related to, the ascending reticular activating system.

The aim of the study presented in this report was to determine whether the electrocorticogram could be activated by electrical stimulation of the head of the caudate nucleus in cats.

Previous findings are contradictory: Gerebtzoff (1941) found that cortical activation-desynchronization could be induced from the striatum. Starzl, Taylor and Magoun (1951), in a study of the diencephalic extension of the reticular activating system, stated that the internal capsule rather than the basal ganglia appeared to be the excitable focus. Later investigations (Shimamoto and Verzeano 1954, Stoupel and Terzuolo 1954) agreed with Gerebtzoff that electrocortical arousal could be elicited by stimulation of the caudate nucleus.

In a previous study it was shown that cortical potentials which had been ascribed to stimulation of the caudate were in fact elicited from areas outside the caudate (LAURSEN 1961). Spread of stimulating current might also be responsible for electrocortical arousal induced from the caudate. I am reporting a study in which multilead stimulation technique was used to delineate areas in the region of the head of the caudate from which electrocortical arousal could be obtained.

Material and Method

Eleven healthy, mature cats weighing between 2 and 5 kg were prepared as encéphale isolé under ether anesthesia, five by an open operation and six by intraspinal injection of 0.1 to 0.2 ml of tetracaine or alcohol at the level of C. 1. Wounds and pressure points were locally anesthetized with tetracaine.

Two types of stimulating electrodes were used:

a) A 14-lead electrode (BUCHTHAL, GULD and ROSENFALCK 1957) adapted for use in the brain (LAURSEN 1961). The diameter of the electrode was 1 mm, the diameter of the leads was 0.1 mm and the distance between the leads was 0.4 mm. Stimulation was carried out between pairs of adjacent leads. The method of insertion of the multilead electrode and the method for determination of its position have been described (LAURSEN 1961).

b) A monopolar steel needle electrode was used for comparison. Its diameter was 0.8 mm including two coats of laquer. Its pointed tip was bare due to shrinkage of the laquer during hardening at 60° C for 12 hours. A clip in the edge of the wound was used as indifferent electrode. The needle electrode was inserted stereotactically in the brain and its position was determined after the experiments by the method of MARSHALL (1940). The brains were prepared for histological examination using frozen sections cut at 20 μ and stained with thionine.

To reduce artefact, the rectangular pulses of 1 msec duration and frequencies from 50 to 300/sec were delivered through a double screened transformer (BUCHTHAL, GULD and ROSENFALCK 1955). The stimulating currents were varied between 0.2 and 2.5 mA.

Two types of recording were used:

a) To study the electrocorticogram (EEG) during stimulation it was necessary to use a recording system with a sufficiently high frequency response to ensure detection of low voltage artefact from 150—200/sec stimulation. High gain difference amplifiers (GULD)[1] with a frequency response down 3 db at 10,000 and 1/sec were used and the signals were displayed on one beam of a two beam cathode ray oscilloscope the other beam being used for marking of the stimulus. The standing spots were photographed on moving film. Stimulus artefact was eliminated from the record by leading off between two silver ball electrodes resting on the pia in the positions which were found to give least interference. The exposed brain was covered with a pool of paraffine oil and maintained at a temperature of 37° C.

b) To study the distribution of evoked changes in the EEG, six silver wires 0.5 mm in diameter, in contact with the dura, were cemented into holes in the skull and connected to an electroencephalograph using a screw in the skull over the frontal sinus as indifferent electrode.
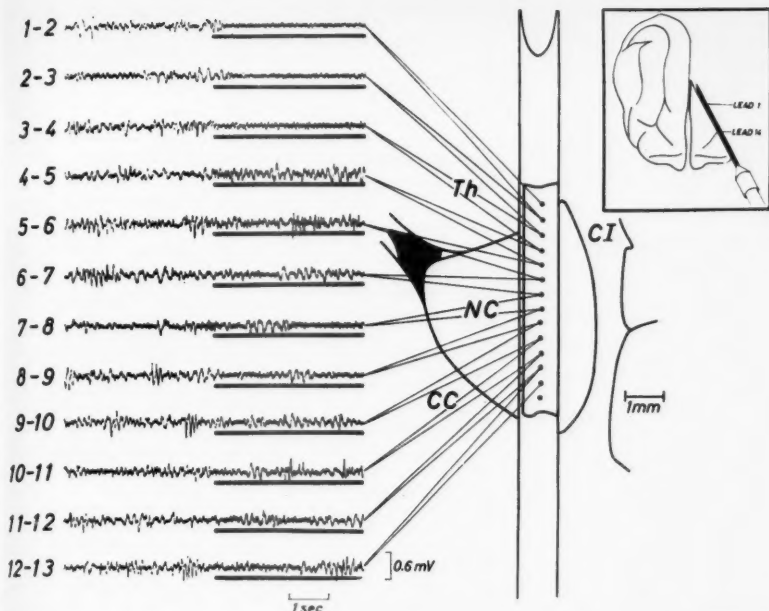
[1] In preparation.

Fig. 1. *Absence of electrocortical arousal during electrical stimulation of the caudate nucleus through a multilead electrode.*

Cat, encéphale isolé. Bipolar recordings (oscilloscope record) from superior suprasylvian gyrus ipsilateral to the side of insertion of the multilead electrode. One msec stimuli were delivered through pairs of adjacent leads at a frequency of 200/sec. The black lines under the records indicate stimulation. Electrocortical arousal was produced by stimulation through lead pairs 1—2, 2—3 and 3—4 situated in the reticular nucleus of the thalamus and the fringe of the caudate adjoining it and not by stimulation through lead pairs 4—13 situated elsewhere in the caudate.

CC Corpus callosum. CI Capsula interna. NC Nucleus caudatus. Th Thalamus.

Results

The head of the caudate nucleus and adjacent structures were explored with the multilead electrode, making only one insertion in each brain to minimize damage.

Low voltage, fast activity was elicited in the EEG during and after 200/sec stimulation through lead pairs situated in the reticular nucleus of the thalamus (Fig. 1, leads 1—2, 2—3). A less pronounced effect was evoked by stimulation through lead pairs situated in a fringe of tissue of the caudate bordering on the thalamus (Fig. 1, leads 3—4) and the effect was probably due to current spread to the thalamus. Stimulation through lead pairs situated in the rest of the caudate did not change the sleep pattern of the EEG (Fig. 1, leads 4—13).

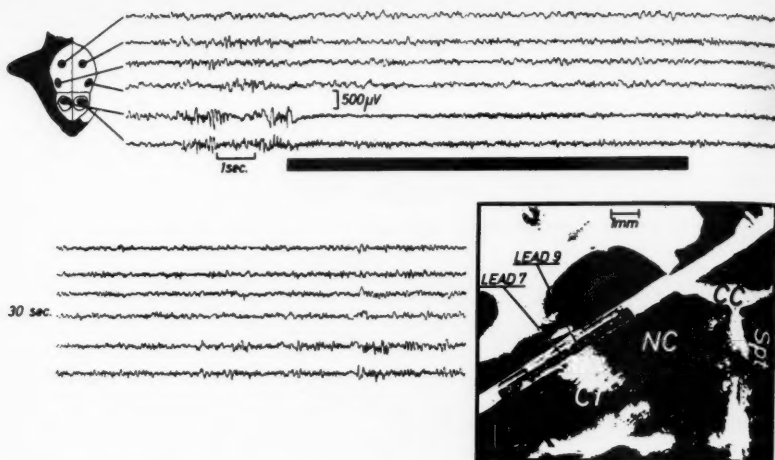When the multilead electrode was inserted in a frontal plane so that the

Fig. 2. *Electrocortical arousal produced by stimulation of the right internal capsule through the multilead electrode* (lead pair 8—9, 1 msec pulses at 150/sec indicated by black line).

An identical response was elicited by stimulation through lead pair 7—8. Stimulation through the other lead pairs did not elicit electrocortical arousal. Cat, encéphale isolé. Extradural unipolar recording. Recording sites shown to the left. Stimulation sites shown on the photograph to the right. The strength of stimulation was 2.5 times the threshold for ipsilateral, pericruciate arousal. The flattening outlasted the stimulus by 40 sec and was most pronounced in the ipsilateral, pericruciate region, slightly less pronounced in the contralateral pericruciate region and just detectable in the other leads.

CC Corpus Callosum. CI Capsula interna. NC Nucleus caudatus. Spt Area septalis.

internal capsule was explored as well as the caudate, electrocortical arousal was elicited only by stimulation through lead pairs situated within a small region in the internal capsule (Fig. 2, leads 7—8, 8—9). Threshold stimulation (approximately 0.3 mA) aroused the EEG in the ipsilateral pericruciate region. With progressively increasing stimulus intensity also the contralateral pericruciate region and the rest of the cortex were activated.

The failure to find electrocortical arousal during stimulation of the head of the caudate was not due to damage produced in it by the multilead electrode: i) Electrocortical arousal was elicited from the thalamus, where the damage produced by the electrode was the same as in the caudate. ii) The absence of electrocortical arousal was confirmed when a unipolar needle electrode was used to stimulate the caudate. This electrode inflicted a minimum of damage due to the small volume of its pointed tip. Stimulation of the reticular nucleus of the thalamus through this electrode elicited diffuse, bilateral electrocortical arousal (Fig. 3 A). Stimulation in the center of the caudate at the same strength and at a strength up to three times the threshold found in the thalamus did not evoke electrocortical arousal (Fig. 3 B).
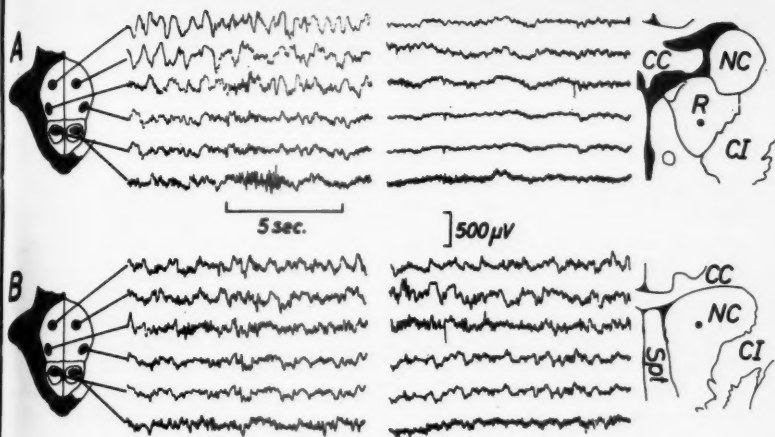
Fig. 3. *Absence of electrocortical arousal (B) during and after stimulation of the caudate through a unipolar electrode.*

Electrocortical arousal (A) with the electrode situated in the left reticular nucleus of the thalamus. Cat, encéphale isolé. Extradural, unipolar recording. Recording sites shown to the left, stimulation sites indicated by black spots in the drawings to the right. The interruption in the middle of the records indicates an interval of 15 sec. (10 sec of stimulation with 1 msec pulses at 150/sec and 5 sec for recovery of the overloaded amplifier). Stimulus intensity 1.3 times threshold.

CC Corpus callosum. CI Capsula interna. NC Nucleus caudatus. R. Nucleus reticularis thalami. Spt. Area septalis.

It has been reported that electrocortical arousal can be elicited from the caudate even in animals anesthetized with barbiturates (SHIMAMOTO and VERZEANO 1954). In the experiments reported here, the low voltage fast activity produced after stimulation of the thalamus and the part of the caudate adjoining it could no longer be elicited immediately after the intravenous injection of small doses of nembutal (7.5 mg/kg).

Discussion

The ascending reticular activating system (MORUZZI and MAGOUN 1949) can be defined as the subcortical regions from which electrocortical arousal can be induced by electrical stimulation excluding the classical sensory systems. The findings presented in this study show, that the caudate nucleus is not a part of this system. This is in agreement with the results of STARZL et al. (1951) but contrary to the results of other authors (GEREBTZOFF 1941, SHIMAMOTO and VERZEANO 1954. STOUPEL and TERZUOLO (1954) and inconsistent with the view recently expressed by JUNG and HASSLER (1960 p. 913) that the "higher extrapyramidal centers" and the "nonspecific activation system" are closely

related. Failure to find electrocortical arousal during stimulation of the caudate nucleus extends previous findings (LAURSEN 1961), that the caudate has no influence on cortical electrical activity.

The electrocortical arousal response ascribed to stimulation of the head of the caudate nucleus was thought to be mediated by a subcortical relay (STOUPEL and TERZUOLO 1954), more specifically the intralaminar thalamic nuclei, because lesions here abolished the response (SHIMAMOTO and VERZEANO 1954). The alternative explanation emerging from the findings presented here is, that it was in fact spread of stimulating current from the caudate to the thalamus which elicited the electrocortical arousal response.

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Heparin and Thrombocytopenia in Experimental Burn Injuries

By

Stig-Arne Johansson

Abstract

Johansson, S.-A. *Heparin and thrombocytopenia in expermental burn injuries*. Acta physiol. scand. 1961. *53*. 239—246. — In experimental burns a decrease in the number of circulating platelets with an ensuing decrease of 5-hydroxytryptamine (5-HT) in whole blood was found. In lung tissues an increase of 5-HT was detected. Pretreatment of the animals with heparin prevented these effects. Coagulation time measurements showed a prolongation of the coagulation time. An increase of the anti-thrombic substances in blood was found. It is suggested that heparin, being a strongly acid polyelectrolyte, acts on the platelets and their ability to absorb and release 5-HT and prevents the onset of a transient intravascular coagulation during burn injuries.

Burn injuries in rabbits are accompanied by a decrease in the number of circulating platelets with an ensuing release of 5-hydroxytryptamine (5-HT, serotonin) (Johansson 1960 a). In anaphylactic reactions the decrease in platelet count is prevented if heparin is given prophylactically before the shock dose (Johansson 1960 b). Survival times of burned dogs treated with heparin is prolonged in comparison to those of untreated dogs (Elrod et al. 1951, Hallberg and Pitzele 1959). As to the mechanism of the protective action of heparin in burn injuries nothing is known.

The purpose of the present investigation is to study whether heparin prevents the decrease in platelet count and inhibits the release of 5-HT in burn injuries.

Methods

Rabbits of both sexes weighing 2.5—3.0 kg were used in these experiments. The animals (including controls) were anesthetized with nembutal (20 mg/kg). About 20 per cent deep third degree burns were produced with hot water (65° C) in 2 min on 20 shaved animals. Ten animals received 50 mg heparin Vitrum containing 102 i. u. per
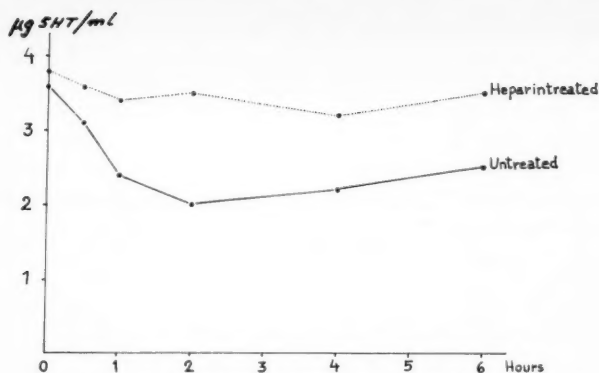
Fig. 1 The mean concentrations of 5-HT (μg 5-HT/ml) in whole blood from 5 untreated and 5 heparin treated animals at intervals after the burn injury. Untreated animals ._____. Heparin treated animals . . . .
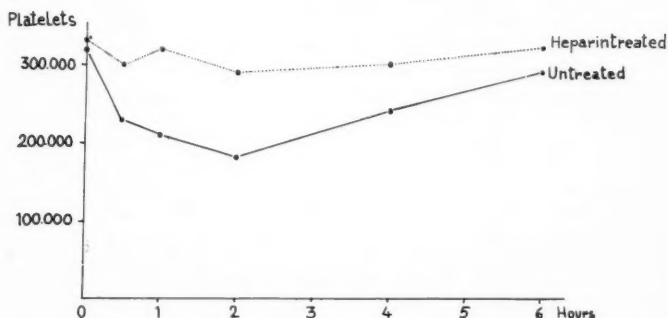

Fig. 2 Average platelet counts in whole blood from 5 untreated and 5 heparin treated animals at intervals after the burn injury. Untreated animals._____Heparin treated animals. . . . .

mg 20 min before the burn injury. Five untreated and five heparin treated animals were killed 90 min after the burn injury and intestines and lungs were homogenized with 0.1 n HCl and their content of 5-HT was determined after extraction into n-butanol. From the remaining ten animals samples of blood were withdrawn through a polyethylene catheter in the carotid artery one hour before and at 30, 60, 120, 240, 360 min after the burn injury. Nine volumes of blood were collected into centrifuge tubes containing 1 volume of 3.8 per cent trisodium citrate ($Na_3 C_6 H_5 O_7 2H_2O$). International Co. lusteroid centrifuge tubes were used. All glassware was silicone treated.

Coagulation time measurements were made according to HEDENIUS (1936). Platelet counts were made by a modified method of KRISTENSON (1937). The anti-thrombic substances were determined according to BLOMBÄCK, BLOMBÄCK and WALLÉN (1955).

The 5-HT concentrations in whole blood and tissues were determined spectrophotofluormetrically after extraction into purified n-butanol according to UDENFRIEND, WEISSBACH and CLARK (1955) and WEISSBACH, WAALKES and UDENFRIEND (1958).

Table I. Platelet counts in thousands per cu. mm before and at intervals after the experimental burn injury

Untreated animals	Before the burn injury	Minutes after the burn injury				
		30	60	120	240	360
Rabbit 1	325	235	250	230	170	—
Rabbit 2	320	150	160	80	—	—
Rabbit 3	315	285	280	275	325	340
Rabbit 4	305	270	255	205	220	245
Rabbit 5	300	190	195	140	—	—
Heparin treated animals	Before the burn injury	Minutes after the burn injury				
		30	60	120	240	360
Rabbit 6	360	310	320	300	310	325
Rabbit 7	340	315	330	315	315	320
Rabbit 8	340	300	320	305	300	300
Rabbit 9	310	300	315	290	305	305
Rabbit 10	300	280	300	265	255	280

Mean value with standard deviation of 50 platelet counts from 5 rabbits anesthetized with nembutal 310 ± 15.

Results

The concentrations of 5-HT and the platelet counts in rabbit blood before and at intervals after the burn injury are shown in Fig. 1 and 2. In experimental burns there occurs a decrease in the total amount of 5-HT in rabbit blood due to a diminution in the number of circulating platelets (Table I). The platelets however, return to normal levels within 6 hours, but the decrease in the 5-HT content of blood persisted for at least 12 hours. In platelet deficient plasma, however, no detectable amount of 5-HT was detected.

In those animals which had been pretreated with heparin the burn injury was accompanied by no decrease or only a slight decrease in the number of circulating platelets and in the 5-HT content of whole blood (Fig. 1—2, Table I).

The amount of 5-HT in intestines was high and because of the wide normal variation a release of the amine from the intestines would be difficult to detect. Analyses from 5 animals one hour after the burn injury gave values within the normal range. An elevation of the 5-HT level in the lung was observed 90 min after the burn injury (Table II). In 5 burned animals treated with heparin no significant increase in the 5-HT content was detected. Three of the unheparinized rabbits died within 6 hours while none of the heparinized animals died within this period (Table III).

Table II. Amount of 5-HT in intestines, lungs and whole blood 90 min after the burn injury to control values from controls. (Range used where the analyses differed by more than 1 μg/g wet tissue. In other cases mean and standard deviations are shown.) (5-HT μg/g wet tissue)

	Controls	90 min after the burn injury	
		Untreated	Heparin treated
Number of animals	5	5	5
Intestine	8—12	8—13	8—14
Lung	2.4±0.3	3.8±0.3	2.6±0.2
Whole blood	3.6	2.3	3.8

Table III. The survival time of heparin treated and untreated animals after experimental burn injuries

	Number of animals	Animals alive after 6 hours	10 hours
Untreated	5	2	1
Heparin treated......	5	5	4

Table IV. The anti-thrombic titre in seconds before and at intervals after the burn injury from 5 untreated animals

	Before the burn injury	Minutes after the burn injury					The animal died after
		30	60	120	240	360	
Rabbit 1 ..	40	142	208	223	92	—	320 min.
Rabbit 2 ..	36	92	102	119	—	—	150 min.
Rabbit 3 ..	36	160	254	265	120	81	Alive after ten hours
Rabbit 4 ..	39	86	93	120	102	66	420 min.
Rabbit 5 ..	37	110	180	116	—	—	220 min.

Table V. The coagulation time before and at intervals after the burn injury

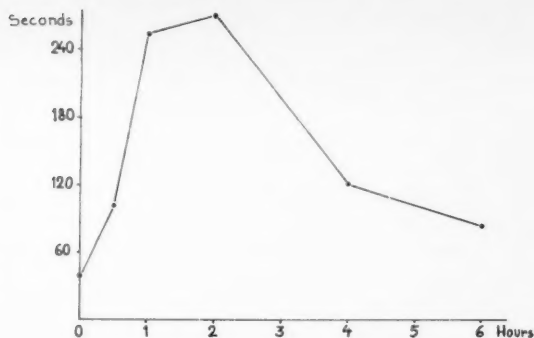| Rabbit	Before the burn injury	Minutes after the burn injury				
		30	60	120	240	360
1	3'50"	8'20"	12'20"	14'10"	>30	—
2	4'15"	7'35"	14'15"	16'30"	—	—
3	3'30"	6'25"	12'00"	11'25"	10'45"	8'05"
4	3'45"	6'50"	7'10"	14'50"	>30	>30
5	4'25"	10'15"	14'50"	16'15"	—	—

Fig. 3. The anti-thrombic titre in seconds at intervals after the burn injury in one of the animals.

To study whether the animals release heparin to the blood after the burn injury, the anti-thrombin titre was determined in 5 unheparinized animals. The results are given in Table IV. This study shows that an anti-thrombic substance, probably heparin, is released in large amounts in the first hours after the burn injury. The activity of the anti-thrombic substance corresponds to between one and two i. u. heparin per ml whole blood. The coagulation time measurements from unheparinized animals are shown in Table V.

Within 30 min of the burn injury there was a prolongation of the coagulation time and this prolongation persisted in the four animals which died within 7 hours of the burn injury.

Discussion

An increasing amount of interest has been displayed in recent in the role of platelets in blood clotting and hemostasis. They supply agents active in both onset and in other phases of the clotting process. They also have the ability to form white thrombi (VAN CREVELD 1954). Finally they contain a vasoconstrictor substance, 5-HT, which is liberated in the coagulation of blood where the platelets are disrupted (ZUCKER and BORELLI 1954). Almost all the 5-HT circulating in the blood is bound to the platelets (HUMPHREY and JAQUES 1954). Deep venous thrombosis and pulmonary embolism are rather common complications of burn injuries (ALLGÖWER and SIEGRIST 1959). A decrease in platelet number has been noted in man as early as a few hours after the burn accident (MAC DONALD et al. 1945) and this study confirms this finding.

After burning there occurs delayed eosinophilia, which might result from sensitisation following the absorption of altered proteins in the burned area (SEWITT 1951). FEDEROV (1956 and 1959) holds that the body is flooded with antigen derived from the burned skin and that this is responsible for part of the burn syndrome as a result of an autoimmunisation reaction.

In rabbits there is a marked diminution in the circulating number of platelets a few hours after the burn injury accompanied by a decrease in the whole blood concentration of 5-HT. Because of the rapid metabolism of liberated 5-HT (JOHANSSON 1960 b), a decrease in the number of circulating platelets thus produces a corresponding drop in the 5-HT content of the blood.

To study whether 5-HT is released from other sources than platelets the content of the amine in intestines and lung tissues was determined. No change in the content of 5-HT in intestines could be detected. An increase in the 5-HT concentration in lung tissues was however found, probably due to increased amounts of platelets in this tissue.

AVDAKOFF in 1876 reported that blood of burned animals was toxic to other animals. Toxic substance from the burned skin or the blood are alleged to produce illness and even death. The experimental findings and the number and variety of substances proposed are voluminous but conflicting. Nevertheless various substances are undoubtedly released or newly formed after burn injuries (SEWITT 1957, ALLGÖWER and SIEGRIST 1957). Experimentally it is important to establish whether in-vivo inhibition of the formation or the release of such substances can reduce the oedema and be useful in the treatment of burn injuries.

Burn injuries are nearly always followed by oedema, although little is known about its exact mode of formation. It is interesting to note, however, that subcutaneous administration of 5-HT causes a marked increase in capillary permeability (ROWLEY and BENDITT 1956). The local oedema producing effect of subcutaneous injections of protein or dextran in rats is inhibited if the animals have been pretreated with the 5-HT liberating substance, reserpine (WEST 1957). 5-HT also increases the permeability of the beet root cell membrane to water-soluble pigments (PICCLES and SUTCLIFFE 1955). Along similar lines PICCLES (1956) found that pretreatment of erythrocytes with 5-HT accelerates their subsequent hypotonic hemolysis. 5-HT thus seems to produce alterations in the permeability of the cell membrane.

The released amounts of 5-HT in rabbit blood after experimental burn injuries are too small to cause death if injected intravenously into rabbits but many of the contributing symptoms which appear might be produced by 5-HT.

ELROD, MC CLEERY and BALL (1951) found that the survival times of burned dogs treated with heparin was twice that of untreated animals. They suggested that the effect was due to improved renal function and resorption of oedema fluid.

The present study shows that an anti-thrombic substance, probably heparin, is released in blood after burn injuries, and that this may be the cause of the prolonged coagulation time measurements. In two rabbits, however, the coagulation time exceeded 30 min, which is too long a time to be caused by the released amount of anti-thrombic substance. It is interesting to note that the rabbit with

the highest platelet count throughout, had the highest anti-thrombic titre and was the only untreated animal to survive.

As to the mechanism of the protective action of heparin in burn injuries nothing is known. In an earlier study it has been shown that heparin has no or very little ability to combine with 5-HT at a pH and a salt concentration corresponding to that in blood (JOHANSSON 1960 b). Heparin thus probably does not act in blood by binding released 5-HT. Thrombocytopenic blood can be kept fluid for hours by the addition of a very small amount of heparin (ALLEN et al. 1947). Whether the platelets absorb heparin on their surface or not is not known. Heparin, however, produces a marked increase in the negative charge of platelets in citrate buffer (ROSS and EBERT 1959). The anti-thrombic effect of heparin is well known. A possible role of heparin in burn injuries would therefore be to prevent the onset of intravascular coagulation. This study shows that large amounts of an anti-thrombic substance, probably heparin, is released into rabbit plasma during burn injuries. Preliminary results from other investigations have shown that protrombin-proconvertin and fibrinogen are decreased after the burn injury (JOHANSSON 1961). Transient intravascular coagulation after burn injuries might then be responsible for part of the appearing shock symptoms and death in burn injuries. Heparin thus may have a protective action in burn injuries by its direct action on platelets and its influence on different humoral coagulation factors.

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Modifications of the Febrile Response to Pyrogen by Hypothalamic Heating and Cooling in the Unanesthetized Dog

By

H. T. Andersen[1], H. T. Hammel and J. D. Hardy

Abstract

Andersen, H. T., H. T. Hammel and J. D. Hardy. *Modifications of the febrile response to pyrogen by hypothalamic heating and cooling in the unanesthetized dog.* Acta physiol. scand. 1961. *35.* 247—254. — Experiments have been carried out with the aim of obtaining additional information on the mode and site of action of pyrogens. On the assumption that these agents act on the thermally sensitive cells of the anterior hypothalamus by raising the "set point" for physiological temperature regulation to a higher level, it was planned to counteract their effect by heating this region of the hypothalamus with 3.7 megacycle radio frequency energy between implanted thermodes, or enhance their effect by cooling the same area by circulating water through the thermodes. Temperatures were measured by means of thermocouples inserted into the thermodes. The rectal and the skin temperatures were also recorded by means of thermocouples. Fever was induced in the animals by intravenous administration of a pyrogenic polysaccharide obtained from Pseudomonas. The body temperature started to increase 30—45 minutes after the injection and usually remained at the elevated level for 3 hours or more. Heating of the hypothalamus was performed 1) immediately after administration of pyrogen, 2) while the fever developed and 3) when the fever condition was maximal. In the first case, the fever failed to develop, in the second instance, the rise in rectal temperature was stopped, and in the third event, thermoregulatory responses similar to those obtained with the same technique at normal body temperature were elicited. In the experiments in which the pyrogenic action was enhanced by hypothalamic cooling a "hyperfever" was produced.

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The site and mode of action of bacterial pyrogens remain a question of considerable controversy, although much work on the problem has been performed by several workers.

Fevers induced by the administration of pyrogenic substances involve both an inhibition of the mechanisms subserving heat loss, and a stimulation of those regulating heat production (PARK and PALMES 1948, THOMPSON 1959).

LIEBERMEISTER (1875) suggested that fever is caused by an upward displacement of the "set point" temperature at which the centers controlling body temperature regulate. This resetting hypothesis was adopted and developed further by DU BOIS (1941).

CHAMBERS et al. (1949) studied the febrile response to bacterial pyrogens in normal cats and dogs, as well as in preparations with different lesions in the central nervous system. They found that decortication, and thalamic and caudal hypothalamic lesions did not prevent the febrile response, whereas decerebrate preparations with most of the midbrain intact failed to show such an effect. GRANT (1949) found no evidence that pyrogenic substances influence the hypothalamic temperature regulating centers. He suggested that their action is one of interference with motor mechanisms of the lower levels of the brain stem.

HALL et al. (1951) stated plainly that the action of bacterial pyrogens is not a resetting of the primary thermoregulatory centers of the hypothalamus, but that the thermostatic disturbances observed are elicited from a general interference of these substances on the autonomic functions controlled by the brain stem. THOMPSON (1959) on the other hand, showed very convincingly that dogs in which the grey matter of the posterior hypothalamus had been completely extirpated, were unable to develop a response to bacterial pyrogens.

Further information on the site of action of the bacterial pyrogens would probably be derived if their action could be counteracted or enhanced by respectively heating or cooling the thermosensitive cells of the hypothalamus during various phases of the developing fever. The results of such experiments are reported in this paper.

Material and Methods

Four mongrel dogs were used for the experiments. Fever was induced by intravenous administration of "Piromen"[1].

Because dogs develop a refractoriness towards the pyrogenic action, only 2—3 exp. were performed on each individual with 2—4 weeks interval between runs. The initial dose was 0.6 μg/kg body weight. Subsequent doses were increased by 0.2 μg/kg body weight in each additional experiment. These doses invariably caused a fever of roughly 1° C, and because of the moderate dosage, the side-effects, retching, vomiting and defecation were only observed in 2 exp. The pyrogenic action of the bacterial polysaccharide was counteracted by heating the anterior hypothalamus with 3.7 megacycle radio frequency energy, and enhanced by cooling of the same area with circulating

[1] Pseudomonas polysaccharide manufactured by Travenol Laboratories, Inc., Morton Grove, Ill., U. S. A.
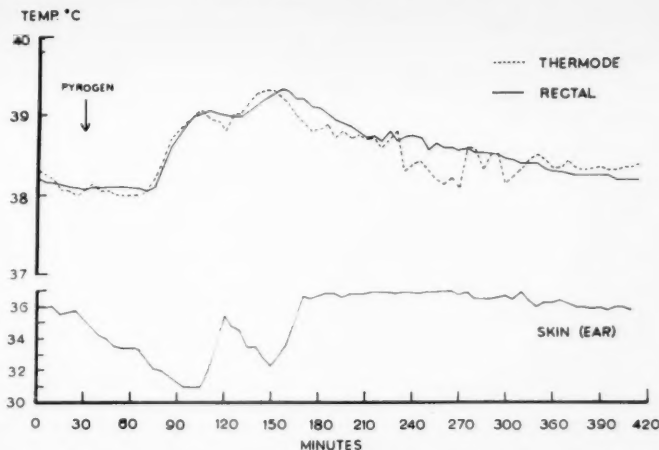
Fig. 1. Normal development of pyrogenic fever. The usual two-step rise in rectal and hypothalamic temperature is shown. Note cutaneous (ear) vasodilatation between steps.

water. The rectal temperature was used to indicate the febrile response, and the ear temperature was measured for an indication of vasomotor activity. In the experiments in which the hypothalamus was heated the temperature of the thermodes were recorded, and in the experiments where cooling was employed both the stimulating and the hypothalamic temperatures were similarly measured. In these latter experiments an average skin temperature was also recorded. The details of the techniques employed have been described elsewhere (HAMMEL *et al.* 1960, ANDERSEN *et al.* 1960).

The following types of experiments were carried out:

A. Heating of the hypothalamus 1) immediately after administration of pyrogen, 2) while the fever developed and 3) when the fever condition was maximal.

B. Cooling of the hypothalamus during the development of pyrogenic fevers so that the temperature of the hypothalamus was kept well below the rising rectal temperature.

All experiments were carried out at environmental temperatures of 27—28° C.

Results

Normal development of pyrogenic fever

A typical development of the febrile response to i. v. administration of Piromen is shown in Fig. 1. The thermode temperature is, in this experiment, the temperature of the anterior hypothalamus. The familiar two-step development of the pyrogenic fever which has been noticed by several investigators (CHAMBERS *et al.* 1949, THOMPSON 1959) is clearly seen. The period of time which elapsed from the administration of pyrogen until the rectal and hypothalamic temperatures started rising was 35 to 45 min. The ear temperature mostly fell during this "latency-period". One very interesting feature of this curve is that in the normal development of pyrogenic fever the hypothalamic temperature was always higher than the rectal, whereas during the first hour of defervescence the hypothalamic temperature was the lower of the two. In
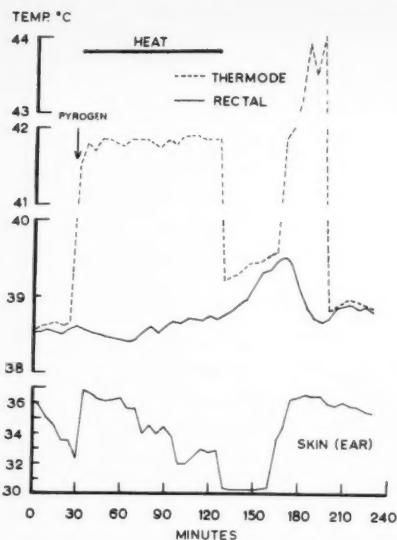
Fig. 2. Inhibition of the febrile response to "Piromen" by heating the stimulating thermodes to 41.8° C after the administration of the pyrogen. The normal response followed immediately after discontinuing the thermal stimulus.

the late stages of defervescence the hypothalamic temperature commonly started fluctuating around the rectal. Shivering was invariably exhibited during the fever development, and panting likewise during defervescence.

Hypothalamic heating immediately after administration of pyrogens

In order to inhibit the development of fever after the administration of pyrogen, the anterior hypothalamus was heated as soon as Piromen had been injected. The result is presented in Fig. 2. The ear temperature rose in response to the heating, and the rectal temperature fell slowly. After a normal latency of 45 min, a period followed during which the ear temperature fell throughout. The animal exhibited a small rise in the rectal temperature, but only enough to bring it up to 0.2° C above that of the period before pyrogen was administered. During the continued heating of the hypothalamus this level of rectal temperature was maintained without significant changes for 45 min. When the heat stimulation of the hypothalamus was cut off, the ear temperature fell abruptly. The rectal temperature rose rapidly 1.0° C above the resting level. By heating the anterior hypothalamus to a higher temperature than before it was possible to induce cutaneous vasodilation as seen from the ear temperature, and a precipitous fall in the rectal temperature. When the heating was stopped, the rectal temperature increased by 0.3° C and then levelled off.

Inhibition of a developing fever by hypothalamic heating

Another experiment is shown in Fig. 3. Pyrogen was given, and the thermode temperature was kept only 1.5° C above the rectal temperature. Due to this
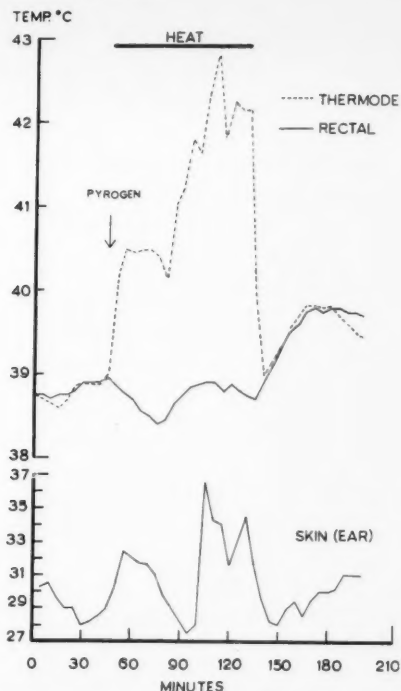
Fig. 3. Inhibition of pyrogenic fever during its development due to hypothalamic heating. The febrile response was obtained upon discontinuation of the thermal stimulus.

slight stimulation, the ear temperature indicated vasodilation, and the rectal temperature fell roughly 0.6° C. Thirty min after the injection the ear temperature started decreasing, the fever developed accompanied by shivering, and the hypothalamic temperature fell in spite of unchanged stimulation. The rectal temperature increased rapidly. By increasing the stimulating temperature of the thermode to 42—43° C it was possible to inhibit the developing fever and lower the rectal temperature due to vasodilation (see ear temperature) and panting. Stoppage of the heat supply to the hypothalamus brought about vasoconstriction, vigorous shivering and an abrupt rise in rectal temperature to 1.2° C above the resting.

Effect of hypothalamic heating on fully developed pyrogenic fever

Fig. 4 shows an experiment in which the fever was allowed to develop normally. When a stage had been reached at which the rectal temperature levelled off after having increased 1.0° C above normal, the anterior hypothalamus was heated as shown. The responses elicited were strong cutaneous vasodilation and a marked fall in rectal temperature of 0.6° C. Removal of the stimulus caused vasoconstriction, shivering and a second rise in rectal temperature almost to the same level as the first. The same sequence of events followed
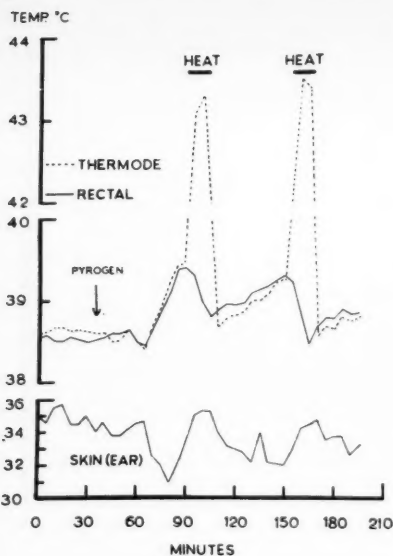
Fig. 4. Hypothalamic heating applied in the case of fully developed pyrogenic fever. The thermal responses elicited were very similar to those obtained at normal body temperature.
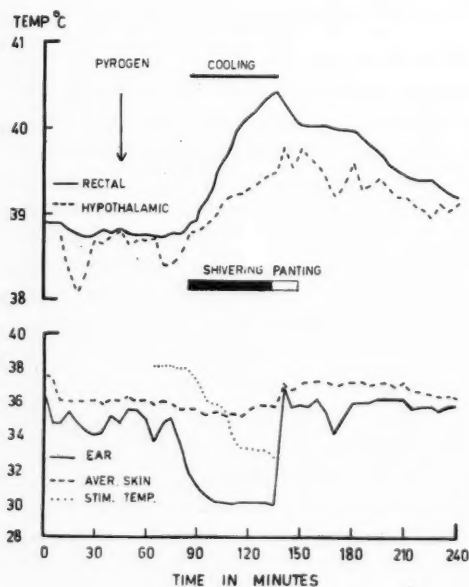

Fig. 5. The additive effect of injected pyrogen and hypothalamic cooling. A "hyperfever" was produced.

repeated stimulation, and a third rise in rectal temperature was produced after the heating had been discontinued. This shows that the pyrogenic substance was exerting its effect throughout the experiment.

Hypothalamic cooling and pyrogenic fever development

In the experiments in which the anterior hypothalamus was cooled after the pyrogen had been given, a summation of these two stresses was obtained. The result, an elevation of the rectal temperature well above the level corresponding to the pyrogen alone, is demonstrated in Fig. 5. As soon as the cooling was stopped, the ear temperature increased 7° C, and the animal started panting. The average skin temperature also rose conspicuously. This situation, however, lasted for only 10—12 min, during which the rectal temperature fell from 40.4° C to 40.0° C. The rectal temperature levelled off at this point and remained almost constant for 35—40 min before the onset of defervescence. The hypothalamic temperature also rose during the stages in which the fever developed. This was inevitable in order that the temperature of the circulating water should be held within relatively physiological limits (38—33° C). An experiment was performed where the hypothalamic temperature was kept almost constant, but to obtain this constancy the stimulating temperature had to be lowered to 26.8° C. The findings in this latter experiment were essentially the same as those reported in Fig. 5, except that a peak of 41.1° C in rectal temperature was reached. After abolishing the cold stimulation, the rectal temperature due to the pyrogen alone was found to be 40.6° C.

Discussion

The negative or positive summation of an injected pyrogenic substance and hypothalamic heating or cooling respectively during the febrile response has been demonstrated by the experiments reported in this paper. The findings that it is possible to suppress the fever from developing by heating the thermosensitive structures in the anterior hypothalamus, and likewise to enhance the response to the pyrogen by local cooling of this same area, may be interpreted as indicative of the correctness of the thermostatic resetting theory. Especially because the thermal stimuli employed have been moderate, it seems probable that the pyrogen administered acts on the physiological thermostat directly. Hence, when this area is locally heated, the induced temperature change satisfies the requirements of the thermostat and prevents general fever development. Furthermore, the hyperfever produced by moderate, local cooling, shows the additive effect of two stresses working in the same direction, and it is quite conceivable that both stimuli are exerting their effect through the thermosensitive structures in the anterior hypothalamus. There can be little doubt, however, that "Piromen" and probably other pyrogens, act at other locations in the central nervous system, as evidenced by the side-reactions, retching, vomiting and defecation which may be observed when larger doses of pyrogen are given.

At the present time one must consider two alternative explanations of our findings in addition to the one outlined above.

1. The pyrogenic substances act on other structures in the central nervous system in addition to the thermoregulatory cells in the anterior hypothalamus. Displacement of the temperature of the latter will, however, respectively counteract or enhance the effect exerted on these structures as described.

2. The pyrogenic substances act exclusively on central nervous structures other than physiological thermostat, but in such a way that the result is a general rise in body temperature. In this case one is, in our experiments, simply left with two different physiological stimuli which have different sites and modes of action, but which will summate algebraically in their thermal effect.

It seems, however, that these two alternative theories are rendered less likely by the results reported in the present study, because the stimulating temperatures employed were about the same magnitude as used to produce the same thermoregulatory effects in the normal dog. Especially if alternate theory no. 2 is correct, one would expect that the amount of hypothalamic heating needed to counter the fever drive from extra-hypothalamic origin would be larger than it was actually found to be.

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Augmentation of Cardiac Contractile Force and Heart Rate by Medulla Oblongata Stimulation in the Cat

By

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Abstract

Rosén, A. *Augmentation of cardiac contractile force and heart rate by medulla oblongata stimulation in the cat.* Acta physiol. scand. 1961. *53.* 255—269. — Electrical stimulation in two relatively well circumscribed regions of the medulla oblongata elicited tachycardia and augmentation of the right ventricular contractile force recorded by a strain gauge arch technique. One of the two regions was found to be situated within the more central part of the pressor area. Stimulation in this region generally produced vasoconstriction in skeletal muscle parallel with the increased cardiac activity. The second region was found to be located in the "sympathetic vasodilator area" in the ventrolateral part of the medulla. Muscle vasodilatation elicited by stimulation in that region was accompanied by an increase in the cardiac functions. The augmentation of cardiac activity with stimulation of the two regions was mediated by the sympathetic innervation of the heart and, to a minor extent, by an elevated secretion from the adrenal medulla.

A tachycardia following stimulation in the medulla oblongata has been reported to occur by *e. g.* Chen *et al.* (1936), Monnier (1939), Mc Queen *et al.* (1954), Amoroso, Bell and Rosenberg (1954) and Peiss (1960). However, the literature contains very few data on augmentation of the cardiac contractile force with such stimulation. Peiss (1958) observed, on stimulation in the pressor area, alterations of pulse pressure which were interpreted as signs of an increased force of myocardial contraction. The finding of Randall and Rohse (1956) that stimulation of the cardiac sympathetic nerves increases the force of

255

cardiac contraction without necessarily raising the heart rate suggests that separate fibers exist for these two functions. While the hypothalamic representation of the inotropic fibers to the heart has been subjected to relatively thorough study (RUSHMER, SMITH and FRANKLIN 1959, SMITH et al. 1960, ROSÉN 1961 a), their representation in the medulla oblongata has been treated only in the above mentioned investigation of Peiss.

The present investigation was designed to establish whether the sympathetic vasodilator outflow which passes through the ventrolateral portion of the medulla (LINDGREN and UVNÄS 1953, LINDGREN et al. 1956), is accompanied by fibers, the stimulation of which produces cardiac inotropic and chronotropic effects. Electrical stimulation of the vasodilator pathway in the central nervous system elicits, in the cat and the dog, a cholinergic sympathetic skeletal muscle vasodilatation. Parallel with the vasodilator response, increased liberation of catechol amines from the adrenals occurs (GRANT et al. 1958, LINDGREN, ROSÉN and UVNÄS 1959a). Hypothalamic stimulation of the vasodilator system causes, in addition, excitation of inotropic and chronotropic fibers to the heart (ROSÉN 1961a). The question arose whether the concomitance of muscle vasodilator and of cardiac responses on stimulation in the hypothalamus was purely coincidental or whether it signified an interrelationship. Should the vasodilator outflow prove to be accompanied by cardio-active fibers even at other levels of its central course, the possibility of an interrelationship would be strengthened. The vasodilator outflow in the medulla oblongata is well suited for such complementary studies, for in that part of the brain stem its course is relatively well separated from those of other vasomotor regulating structures. — The pressor area will also be studied with respect to the occurrence of cardio-active structures, since it would be of interest to verify by direct recording of the myocardial contractile force, Peiss's finding — in studies of blood-pressure variations — that an increased cardiac contractile force frequently accompanies stimulation in the pressor area.

Method

Twenty-five successful experiments were performed. The cats, weighing between 1.7 and 3.7 kg, were anesthetized with chloralose (25—50 mg/kg) and urethane (200—500 mg/kg). The rectal temperature was maintained at 37° C with the aid of an infrared lamp directed towards the abdomen and thorax.

The arterial pressure was measured with a Statham transducer (P 23 A) via a plastic cannula inserted into a carotid artery and advanced so that its tip was situated in the aortic arch. The muscle blood flow was recorded in the right femoral artery of a skinned leg. In order to exclude blood flow from the paw a tight ligature was placed just above the ankle. The blood flow in the femoral artery was conducted, via a plastic cannula, to a silicone-filled drop chamber (LINDGREN 1958) where the number of drops was registered by an ordinate recorder via a photocell. For the maintenance of warmth and moistness the detached skin was replaced around the muscle. To prevent coagulation, heparin (5 per cent, Vitrum) was administered intravenously in a dose of 25 mg/kg. Dextran (Macrodex, Pharmacia) was given intravenously in doses sufficient to com-

pensate blood losses. Positive-pressure artificial respiration was maintained during the experiments. In 12 animals bilateral cervical vagotomy was done before the experiment, and in 8 of these that carotid artery which had not been cannulated for blood-pressure measurement was occluded prior to bulbar stimulation and remained occluded throughout the experiment, in order to eliminate any cardiac effects which might have arisen via baroceptor mechanisms.

The Horsley-Clarke technique was used for stimulation. The skull was perforated with a dental drill so that an electrode could be introduced in the medulla on the right side. Both unipolar (6 experiments) and bipolar (19 experiments) electrodes were employed. The unipolar electrode was 0.45 mm in diameter and had a pointed, uninsulated tip 0.3 mm long. The bipolar electrode consisted essentially of two unipolar electrodes the uninsulated tips of which had been so ground as to form flat oblique surfaces, one reaching 0.5—0.7 mm deeper than the other (conforming in principle to the illustration under B, Fig. 2, page 36, LINDGREN 1955). Electrical stimulation, 1.5—2.5 V, 70 cps, pulse duration 2 msec, was produced by a square-wave generator.

The two stellate ganglia were carefully exposed via an approach through the thoracic wall between the first and second rib on each side. The thoracic wall was thereupon closed. It was later reopened for removal of the ganglia, then closed again. The adrenals were mobilized via an abdominal wall incision for later ligation and removal when required.

The right intraventricular pressure was recorded, in 14 experiments, by means of a Statham transducer (P 23 A). Following thoracotomy a polyethylene catheter was advanced through the ventricular wall into the cavity with the aid of a pointed metal guide which was then removed. The heart rate was measured with an interval recorder (GOLDSCHMIDT and LINDGREN 1961) via impulses from the blood-pressure channel of a Grass (Polygraph) instrument.

The myocardial contractile force was measured with a strain gauge arch. Following thoracotomy and incision of the pericardium the strain gauge arch was attached, in all experiments, to the right ventricular wall by means of sutures which penetrated deeply in the heart muscle. The sutures were so placed as to avoid traumatization of any major coronary vessel. The muscle segment between the two points of attachment was conventionally stretched 40—50 per cent, as described by COTTEN and BAY (1956), in order to minimize those disturbances in recording which attend enlargement of the ventricle. The thoracic wall was subsequently closed as far as possible for the maintenance of warmth and moistness. Throughout the recording the intrathoracic pressure was atmospheric.

In 7 experiments use was made of an open strain gauge arch similar in essentials to that described by BONIFACE, BRODIE and WALTON (1953). For the remaining 18 experiments a somewhat modified, and far more sensitive arch (ROSÉN 1961 a) was employed. For further details of and comment on the strain gauge arch technique in measurement of cardiac contractile force, reference should be made to previous investigations (COTTEN 1953, COTTEN and BAY 1956, ROSÉN 1961a).

Results

Cardiac Responses to Stimulation in the "Sympathetic Vasodilator Area"

The stimulation took place, in all experiments, within that area of the sympathetic vasodilator outflow which LINDGREN et al. (1956) observed in the ventrolateral portion of the medulla oblongata. In 9 experiments such stimulation elicited vasodilatation in the skeletal muscles. That the effect was a

Table I. Responses of cardiac contractile force (C. F.) and heart rate (H. R.) to bulbar stimulation of the sympathetic vasodilator pathway eliciting a muscle vasodilator response

	C. F.	H. R.	After adrenalectomy		After stellatectomy	
			C. F.	H. R.	C. F.	H. R.
Cat no. 1 A	+++	+				
» » 2 A	increase[1]	+				
» » 3 V	+	+++	+	+		
» » 4 V, PCO	++	+	+	+		
» » 5	+++	+	+++	+		
» » 6 V	++	+++	++	+++	0	0
» » 7	+++	++	++	+	0	0
» » 8 V, PCO	++	+			0	0
» » 9	+	+			0	0

[1] No adrenaline injected.

A = Adrenalectomized.
V = Vagotomized.
PCO = Persistent carotid occlusion.
C. F. +++ correspond to the response of about 1.0 μg/kg adrenaline i. v.
    ++   »   » »   »   »   »   0.5   »   »   »
    +   »   » »   »   »   »   0.2   »   »   »
    0   insignificant response.
H. R. +++ 15—20 % increase.
    ++   10—15 %   »
    +   5—10 %   »
    0   < 5 %   »   (insignificant response).

cholinergic vasodilator one attributable to the sympathetic vasodilator system was evident from the fact that on stimulation following atropinization it was either substantially reduced or absent. The activations invariably gave rise to an increase in the heart rate and in the cardiac contractile force concomitantly with the vasodilatation (Table I). One of the experiments is illustrated in Fig. 1 (cat 3, Table I). All effects, in this as well as in the remaining 8 experiments, occurred after a maximum latency of 4 sec (for the heart rate, 2 sec). Neither in this nor in other experiments were the cardiac responses affected by atropine. In 4 experiments the right ventricular pressure was recorded. The systolic pressure rose, in each instance, commensurately with the increase of contractile force; the diastolic pressure, on the other hand, remained unchanged.

The magnitude of the cardiac responses is evident from Table I. The augmentation of cardiac contractile force was equivalent to the elevation produced by adrenaline, 0.2—1.0 μg/kg, administered intravenously shortly before or after the stimulation. This method of estimating the responses of the cardiac contractile force was found to be appropriate for experiments of this type (ROSÉN 1961a). The rise in the heart rate on stimulation amounted to between 5 and
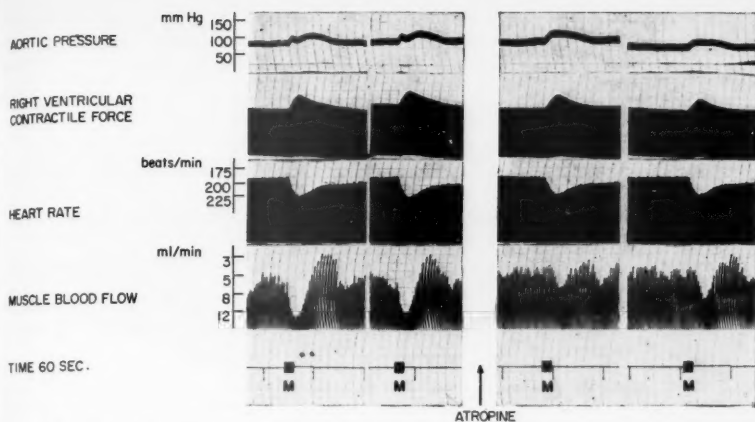
Fig. 1. Cat 3. Aortic pressure, right ventricular contractile force, heart rate and muscle blood flow responses to stimulation in the ventrolateral portion of the medulla oblongata before and after atropinization.
M. Stimulation 2.0 V, 70 cps.
Atropine 0.5 mg/kg intravenously.
Note that there are less increases in muscle blood flow after atropine.

20 per cent. The cardiac responses were mediated generally by the sympathetic innervation to the heart. This finding was corroborated not only by the short latency but by abolition of responses by stellate ganglionectomy. The experiment illustrated in Fig. 2, showing the cardiovascular responses to stimulation of the sympathetic vasodilator tract, is one of the four in which the stellate ganglia were removed (Table I). In this experiment, as in the others, the augmentation of the cardiac responses was practically eliminated by the ganglionectomy. In some experiments bilateral adrenalectomy reduced the cardiac responses to stimulation, but in others it did not. In the former instances catechol amines were evidently released from the adrenals in amounts sufficient to influence the cardiac functions. The amounts liberated in these experiments did not, however, suffice to affect appreciably the vasomotor tonus in the muscles.

During the attempts to activate the sympathetic vasodilator pathway within its circumscribed area in the ventrolateral portion of the medulla, increased cardiac activity was observed to accompany 10 stimulations which produced no significant muscle vasodilatation. In one instance, however, atropine had been administered earlier, and in some experiments stimulation was associated with a minor increase of blood flow which may have resulted from activation of the vasodilator pathway. Five stimulation points were localized histologically, all of them within a region coinciding with the "sympathetic vasodilator area"
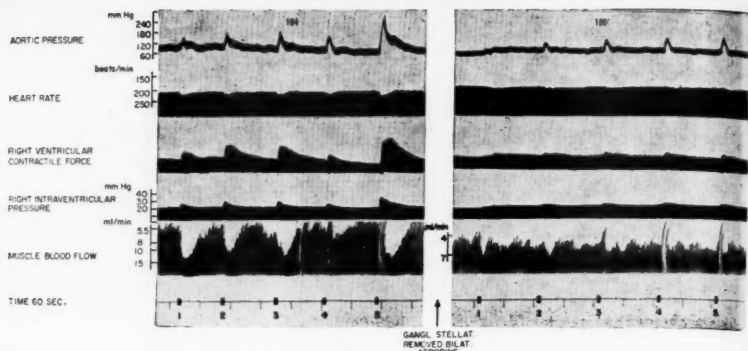
Fig. 2. Cat 8. Aortic pressure, right ventricular contractile force, right intraventricular pressure, heart rate and muscle blood flow responses to stimulation at various levels in the oblongate medulla before and after atropinization and removal of the two stellate ganglia. The adrenals were intact.
1—5: Bulbar stimulations (2.5 V, 70 cps) delivered at five levels approximately 1 mm apart while the tip of the electrode was gradually shifted dorsally from the ventrolateral area. Atropine 0.3 mg/kg intravenously.
Note the minimal cardiac and also the muscle vasoconstrictor responses to stimulation 4, applied above "the sympathetic vasodilator area".

(Fig. 4). The cardiac responses were of the same type and magnitude and had the same latency as those induced by the stimulations which gave rise to vasodilatation (Table II). Adrenalectomy, in the two experiments in which it was done, served to reduce the augmentation of cardiac contractile force (Table II).

Twelve of the experiments were conducted as follows. The electrode was first advanced through the dorsal part of the medulla to the most ventral portion of the ventrolateral area. Stimulation was then produced at different levels, generally separated by 0.5 to 1 mm, the electrode tip being withdrawn a little at a time. It was found that stimulation in a region above the "vasodilator area" in 8 exp. caused no significant changes either in the cardiac contractile force or, in general, in the heart rate; in 2 exp. it reduced the contractile force recording and gave rise to bradycardia concomitant with a substantial blood-pressure fall; and in the remaining 2 exp. it produced an augmentation of the contractile force. In 4 of those experiments in which no cardiac responses attended stimulation above the "vasodilator area", vasoconstrictor responses were observed in the muscles. One of these 4 exp. is illustrated in Fig. 2. The first stimuli elicited vasodilator responses in the muscles, parallel with an augmentation of the cardiac contractile force and a minor tachycardia. Stimulation no. 4 — with the tip of the electrode approximately 1 mm above that in no. 3 — was associated with insignificant cardiac responses and a vasoconstrictor response in the muscles.

Table II. Responses of cardiac contractile force (C. F.) and heart rate (H. R.) to stimulation in the ventrolateral area of the oblongate medulla eliciting no muscle vasomotor response

	C. F.	H. R.	After adrenalectomy	
			C. F.	H. R.
Cat no. 10 V, PCO ..	+	+		
» » 11	++	+		
» » 12	++	++	+	++
» » 13 V, PCO ..	+++	+++		
» » 14	++	+		
» » 15	increase[1]	+++		
» » 16 A	+++	+++		
» » 17	+++	not measured	++	
» » 18	++	not measured		
» » 19 V	+++	+		

[1] No adrenaline injected.

V, A, PCO, C. F. (+++, ++, +, 0) and H. R. (+++, ++, +, 0) see table I.

Cardiac Responses to Stimulation in the More Central Portions of the Pressor Area

In 14 exp. stimulation was applied in the more central parts of the region which ALEXANDER (1946) described as the pressor area. The elicited response consisted, in each instance, of an increased right ventricular contractile force, a heightened systolic pressure (diastolic pressure unchanged) in the right ventricle and, in general, tachycardia. In all except one of these experiments the stimulation was observed to be accompanied by varying degrees of muscle vasoconstriction. One of the experiments is illustrated in Fig. 3, from which it will be seen that the stimulation elicited increased cardiac activity parallel with muscle vasoconstriction (the vasoconstrictor response was most conspicuous at the last stimulation). The augmentation of the cardiac responses was reduced following adrenalectomy. Stimulation plus intravenous injection of adrenaline 1 μg/kg (M + A in the figure) produced effects on the heart similar in amplitude to those obtained prior to adrenalectomy. This suggests that catechol amines had been liberated from the adrenals to approximately the same extent, as the injected amount, and had influenced the cardiac functions. The rise in cardiac responses was nearly abolished following stellate ganglionectomy. The adrenal catechol amines and the sympathetic innervation to the heart were thus both responsible, in this experiment, for the increased cardiac activity. In another experiment with stimulation in the pressor area (stimulation no. 5 in Fig. 2), the cardiac augmentation mediated via the cardiac sympathetic innervation was predominant. The stimulation parameters were the same as those used for activation in the ventrolateral area, though pressor area stimulation gave rise to more
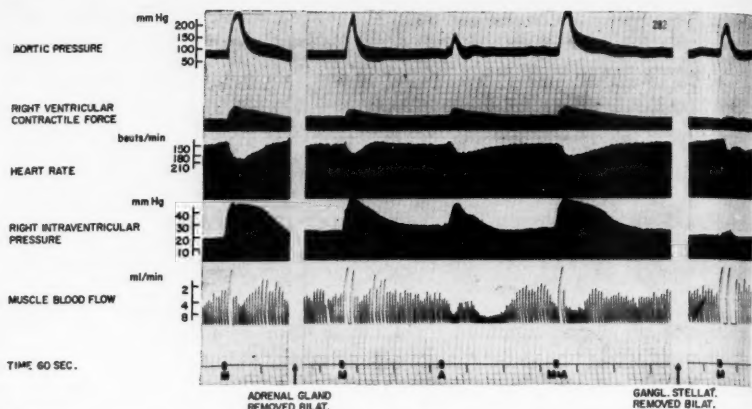
Fig. 3. Cat 21. Aortic pressure, right ventricular contractile force, right intraventricular pressure, heart rate and muscle blood flow responses to stimulation in the pressor area before and after removal of both adrenals and both stellate ganglia.
M. Stimulation 2.0 V, 70 cps.
A. Adrenaline 1.0 μg/kg intravenously.
Note that after adrenalectomy there is a partial, and after stellatectomy an almost total, reduction of the cardiac responses to stimulation.

pronounced cardiac effects (compare Table I and II with Table III). Under the experimental conditions obtaining, the augmentation of cardiac contractile force on stimulation in the pressor area was equivalent to the increase evoked by intravenous injection of adrenaline in a dose of 1 μg/kg or more (Table III). The rise in heart rate varied, in general, between 5 and 20 per cent; in one instance it amounted to 50 per cent.

In 13 of the 14 experiments (the exception being cat 24 in Table III) the latency of the cardiac responses amounted to a maximum of 4 sec (for the heart rate 2 sec). This indicates that the responses were elicited, at least in part, via the sympathetic innervation to the heart. It will be seen from Table III that in the cases with short latency the cardiac responses were substantially reduced or altogether eliminated on stimulation applied following stellate ganglionectomy. A reduction of cardiac contractile force resulted from ganglionectomy in itself, in general, fairly small, though it occasionally amounted to almost 50 per cent. Parallel with that decrease in contractile force there was a minor fall of systolic pressure in the right ventricle as well as moderate bradycardia. In one experiment (cat 24, Table III) approximately 10 seconds elapsed before the cardiac responses were observed. The same was true of cat 13 on stimulation following stellate ganglionectomy. These observations suggest that humoral mechanisms were involved in the responses. In the other experiments too, such

Table III. Responses of cardiac contractile force (C. F.) and heart rate (H. R.) to bulbar stimulation in the pressor region generally eliciting a muscle vasoconstrictor response

	C. F.	H. R.	After adrenalectomy		After stellatectomy	
			C. F.	H. R.	C. F.	H. R.
Cat no. 3 V	+++	+++				
» » 10 V, PCO ..	++++	+++				
» » 11	+++	+				
» » 24 V, PCO ..	+++	+	0	+		
» » 25	+++	+	+	+		
» » 20 V, PCO ..	++++	+++	+++	++	0	0
» » 6 V	+++	increase 50 %	+++	++	0	+
» » 21 V, PCO ..	++++	+++	+++	++	0	+
» » 22 V, PCO ..	++++	++	++++	+	0	+
» » 23 V	++++	++	++++	++	0	0
» » 5	+++	0			0	+
» » 8 V, PCO ..	+++	++			0	0
» » 12 A	+++	++			0	0
» » 13 V, PCO ..	+++	++			++	++

V, A, PCO, C. F. (+++, ++, +, 0) and H. R. (+++, ++, +, 0) see table I.
C. F. ++++ correspond to the response of about 1.5 μg/kg or more of adrenaline i. v.

mechanisms — in addition to the nervous mechanism — may have played a role. In several experiments bilateral adrenalectomy was done prior to stellate ganglionectomy, following which a reduction of cardiac responses was frequently observed (Table III) — a finding which suggested that catechol amines liberated from the adrenals on stimulation were a contributory cause of the increased cardiac activity. It was not possible to determine, however, whether the amount of catechol amines also sufficed to affect the vasomotor tonus in the muscles, since reliable evaluation was precluded by the very marked blood-flow and blood-pressure responses which were also present after adrenalectomy.

Comment

Stimulation in the pressor area gave rise to such marked cardiovascular effects that the heart volume might have been changed. This factor is of particular significance in that a major degree of heart enlargement will affect the strain gauge arch recording. With moderate enlargement, however, the recording will not be appreciably influenced (COTTEN 1953, COTTEN and BAY 1956, ROSÉN 1961a). In all of the present experiments in which both adrenalectomy and stellate ganglionectomy were done, and in some of those in which only one of the two operations was performed, subsequent stimulation caused no significant change in the recording of either the contractile force or the ventricular
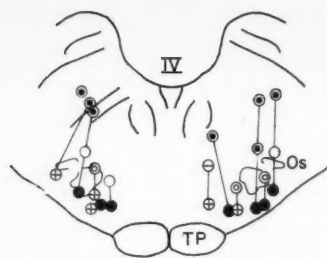
Fig. 4. Drawing of a frontal section through the medulla oblongata of a cat. Stimulation points, histologically localized to a region 2.5 mm anterior or posterior to this section, are represented by the circles.

Os, Superior olive; TP, Pyramidal tract; IV, Fourth ventricle.

⬤ Cardiac augmentation and muscle vasoconstriction.
● Cardiac augmentation and muscle vasodilatation.
⊕ Cardiac augmentation and no muscle vasomotor response.
⊖ Blood-pressure fall, bradycardia and decrease in the recording of cardiac contractile force.
○ No cardiac response, but muscle vasoconstriction.
◎ No cardiac response and no muscle vasomotor response.

The lines connect stimulation points referable to one and the same electrode puncture.

pressure. It follows that any alteration of heart size on such stimulation was not sufficient to influence the recordings. The question remains as to whether stimulation with the adrenals and/or the stellate ganglia intact might have increased the heart volume sufficiently to affect the strain gauge arch recording. As regards the adrenals, WEST and RUSHMER (1957) found that in both unanesthetized and anesthetized dogs intravenous infusion of adrenaline or noradrenaline increased the left ventricular diastolic diameter. Even with large doses of catechol amines the increase seemed, however, to be relatively small. Both adrenaline and noradrenaline, even in small doses, augment the contractile force of the myocardium (e. g. WEST and RUSHMER 1957, ROSÉN 1961b). There is reason, therefore, to surmise that that part of the contractile force response which, in my experiments, disappeared following adrenalectomy was in fact attributable to a direct influence of adrenal catechol amines on the myocardial contractility. As to the stellate ganglia, ANZOLA and RUSHMER (1956) found in dogs that electrical stimulation of the left stellate ganglion in situ actually reduced the dimensions of both ventricles, presumably as a result of augmentation of the myocardial contractile force as well as acceleration of the heart rate. This suggests that on bulbar stimulation in my experiments intact stellate ganglia tended rather to counteract than to facilitate an increase in heart volume. Another observation which may indicate that the strain gauge arch recording was not appreciably influenced by volume changes, is that on bulbar stimulation the right ventricular systolic pressure almost invariably rose — parallel with the increase of contractile force — to not more than approximately double, while the diastolic pressure remained unchanged. Similar right ventricular pressure behavior had previously been observed in seven instances (ROSÉN 1961a, one illustrated in his Fig. 4) of experimentally induced enlargement of the right ventricle, with no significant effect on the strain gauge arch recording from the ventricle.

Histologic Localization

Fifteen cat brains were examined histologically. After removal they were fixed in 10 per cent formaldehyde solution. Frozen serial sections were cut in a plane parallel to the electrode position. Twelve stimulation points were localized in the "sympathetic vasodilator area" (LINDGREN and UVNÄS 1953, LINDGREN *et al.* 1956); 7 of them were associated with vasodilator responses and 5 with no vasomotor responses in the muscles (Fig. 4). An additional 10 stimulation points were localized in the more central regions of the pressor area defined by ALEXANDER (1946) (6 of them are shown in Fig. 4); 9 were associated with vasoconstrictor responses and one with no vasomotor responses in the muscles. Activation of each of these 22 stimulation points had increased the cardiac activity.

Discussion

In the present investigation different regions in the medulla oblongata were stimulated and the resulting changes in the skeletal muscle vasomotor tonus and in the heart rate and cardiac contractile force were studied. It was found that both the myocardial contractile force and the heart rate were increased, apparently independently, on bulbar stimulation in the more central portion of that region which ALEXANDER (1946) described as the pressor area. In general, stimulation at that site elicited — parallel with the cardiac responses — vasoconstriction in the skeletal muscles. In several instances, moreover, stimulation around the ventral border of the pressor area evoked vasoconstrictor responses in the muscles with no demonstrable concurrent changes of cardiac functions. The results confirm PEISS's (1958) claim that the myocardial contractile force may increase on stimulation in the pressor area, and that vasoconstriction elicited by bulbar stimulation is not necessarily associated with cardiac responses. According to my observations the elevated cardiac contractile force arose mainly via excitation of sympathetic fibers to the heart. The fact that activation of the pressor area in my experiments was also attended by cardio-acceleration — likewise via sympathetic fibers to the heart — does not accord with PEISS's observations in his investigation of 1958. He showed, however, in a subsequent investigation (PEISS 1960) that it is possible by pressor area stimulation to induce excitation of chronotropic fibers to the heart. The increased electrical discharge in the inferior cardiac nerve observed by ALEXANDER (1946) on stimulation in the pressor area may — according to my findings — have been due to excitation of both cardio-accelerator and cardio-augmentor fibers.

The results of this investigation do not conflict with the view that the pressor area is concerned in excitation of the entire sympathetic system (CHEN *et al.* 1936, 1937 a, b). Thus it was found that sympathetic vasoconstrictor nerves to muscle blood vessels, sympathetic accelerator and augmentor fibers to the heart, and sympathetic secretion-activating nerves to the adrenal medulla were

excited by stimuli in the pressor area. An increase of the adrenal medullary secretion on stimulation in that area of the medulla oblongata has been described previously (CHEN et al. 1936, 1937b, PEISS 1958, among others). In this connection CHEN et al. found it less likely that normally innervated and unpotentiated organs respond to the liberated catechols. PEISS, on the other hand, thought that with stimulation in the pressor area, catechol amines were released from the adrenals in quantities sufficient to produce distinct vasoconstriction and appreciably elevated cardiac activity. The present results corroborate PEISS's belief that the adrenal medullary secretion may be implicated in the augmentation of cardiac activity which accompanies stimulation in the pressor area. The extent, to which the liberated amounts of catechol amines comes into play in the physiological pattern of reaction, is however still unknown.

In an area of the ventrolateral portion of the medulla oblongata, coinciding with that through which the sympathetic vasodilator outflow passes, stimulation gave rise to excitation of inotropic and chronotropic fibers to the heart, apparently independently of each other, as was the case on stimulation of the vasodilator outflow in the hypothalamus (ROSÉN 1961a). All muscle vasodilator responses to stimulation in the ventrolateral portion were accompanied by an augmented cardiac activity. Since the vasodilator responses failed to occur on stimulation following atropinization, they had evidently stemmed from activation of the sympathetic vasodilator pathway. In some instances stimulation in that part of the medulla oblongata elicited — parallel with the elevated cardiac activity — no vasomotor change in the muscles. It does not necessarily follow, however, that excitation of the vasodilator outflow had been absent in those experiments; the presence of a muscle vasodilator response could have been concealed by simultaneous vasoconstriction. ELIASSON et al. (1951) thus showed that central stimulation in the vasodilator pathway might elicit — parallel with a vasodilator response — a vasoconstrictor effect in the muscles.

It was not previously known that stimulation in the "sympathetic vasodilator area" within the ventrolateral portion of the medulla oblongata causes augmentation of the cardiac contractile force. PEISS (1958, 1960) observed in his investigations the occurrence of tachycardia with a short latency on stimulation in the ventrolateral region of the medulla, and assumed that the heart rate was increased via activation of hypothalamo-spinal pathways. According to my observations it is probable that these stimulations affected the vasodilator outflow. It has previously been demonstrated that in the hypothalamic sector of the vasodilator system vasodilator fibers are accompanied by cardio-active fibers (ROSÉN 1961a). The present investigation shows that such fibers are also involved in the bulbar sector of the vasodilator outflow. According to my results the amount of adrenaline released on bulbar stimulation of the sympathetic vasodilator pathway was not sufficient to influence appreciably the vasomotor tonus in skeletal muscles. Together with the liberated noradrenaline it may, however, have sufficed to increase the cardiac activity. These two

findings confirm other observations (LINDGREN *et al.* 1959b, ROSÉN 1961b). The question, however, still remains to which degree the catechol amines under physiological circumstances contribute to the increased cardiac activity.

LINDGREN and UVNÄS (1955) showed that no anatomical or functional relationship exists between the vasodilator nerves and the pressor area. It has been suggested that the pattern of responses to stimulation of the vasodilator outflow might constitute a functional unit. The question then arises as to the physiologic significance of the vasodilator system.

The muscle vasodilatation and the cutaneous and intestinal vasoconstriction evoked by central stimulation of the vasodilator outflow (ELIASSON *et al.* 1951, LINDGREN and UVNÄS 1953, LINDGREN 1955) tend to produce a shift of blood volume to the skeletal muscles. Redistribution of blood to muscle tissue in order to establish optimal conditions for muscular effort takes place in states of emotional stress and, initially, in muscular work. BLAIR *et al.* (1959) and ABRAHAMS *et al.* (1960) called further attention to the possibility, already discussed by ELIASSON *et al.* (1951) and UVNÄS (1954), that the vasodilator system may be activated in emotional stress. Since the sympathetic vasodilator outflow has a cortical origin (ELIASSON, LINDGREN and UVNÄS 1952) it may be concerned with the initial adjustment of the muscle blood flow during exercise. That hypothesis, however, is not in line with BLAIR *et al.'s* (1961) belief. The cardiac activity is increased both in emotional excitement and, initially, in muscular exercise (for references see ROSÉN 1961a). My observations provide some evidence that the augmentation of cardiac contractile force and the increased heart rate together constitute a functional part of the response pattern of the vasodilator system. This lends further support to the hypothesis that the sympathetic vasodilator outflow is activated in situations such as fear, anxiety, unrest and other emotional states, as well as in the initial phase of muscular work, in order to establish suitable cardiovascular conditions.

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Cardiac Inotropic and Skeletal Muscle Vasomotor Responses to Adrenaline and Noradrenaline. Threshold Doses on Intravenous Infusion

By

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Abstract

Rosén, A. *Cardiac inotropic and skeletal muscle vasomotor responses to adrenaline and noradrenaline. Threshold doses on intravenous infusion.* Acta physiol. scand. 1961. *53.* 270—275. — In cats under chloralose and urethane anesthesia, the mean threshold dose with standard deviation of intravenously infused adrenaline for a cardiac inotropic response was found to be 0.26 ± 0.12 μg/kg/min and for a muscle vasodilator response 0.44 ± 0.20 μg/kg/min. The mean threshold dose of noradrenaline for an inotropic response amounted to 0.37 ± 0.14 μg/kg/min and for a muscle vasoconstrictor response to 1.17 ± 0.45 μg/kg/min. The necessity of caution in estimating the differences in the threshold doses between inotropic and vasomotor responses is emphasized.

Activation of the sympathetic vasodilator outflow by intracerebral electric stimulation elicits — via the sympathetic innervation to the vessels of the skeletal muscles — vasodilatation in the muscles of the dog and cat. In the cat, there is a concurrent increase in the contractile force of the myocardium, produced via the sympathetic innervation to the heart (Rosén 1961 a, b). Furthermore, central stimulation of the vasodilator pathway results in a raised output of adrenaline and noradrenaline from the adrenals (Grant *et al.* 1958, Lindgren, Rosén and Uvnäs 1959 a). On stimulation of moderate intensity, the increase in either of these catechol amines is not, as a rule, sufficient to produce any appreciable vasomotor response in the muscles (Lindgren, Rosén and Uvnäs

270

1959 b). Their raised output does, on the other hand, often suffice to elicit a marked increase in the contractile force of the heart (Rosén 1961 a, b). In the present investigation the threshold doses of intravenously infused adrenaline and noradrenaline required to elicit cardiac inotropic and muscular vasomotor responses were estimated. This was done to determine 1) if the excretion of catechol amines on central stimulation of the vasodilator outflow is — on comparison with the threshold doses for cardiac inotropic responses — sufficient to induce an increase in the contractile force of the heart; 2) if there is a difference in the threshold dose of the catechol amines for a cardiac inotropic and a muscle vasomotor response.

Methods

The experiments were performed on 9 cats, weighing from 2.5 to 3.8 kg, anesthetized with chloralose (25—50 mg/kg) and urethane (200–500 mg/kg). Solutions of l-adrenaline hydrochloride and l-noradrenaline bitartrate in saline were prepared, and placed on ice. All doses are expressed as free base. A polyethylene catheter was inserted in a branch of the left brachial vein. The catheter was connected to a 20 ml syringe, mounted in a constant infusion pump capable of delivering various calibrated infusion rates. In 3 animals, only one rate of infusion was used (1.8 ml/min), with variations in the concentration of catechols in the solution. In the remaining 6 animals, the concentration was constant (10 μg/ml), whereas the rate of infusion varied, but never exceeded 1 ml/min. The infusions were continued until the new levels in the recordings were stable for at least a minute. After cessation of the infusion and the amplitudes in the recordings were back to the initial level another period of generally 2—4 min was allowed to pass until next infusion started. The increase of dose from one infusion to the next was in the range of 79—100 per cent. Due to spontaneous fluctuations a 10 per cent increase in the contractile force was considered to be the threshold response. For the same reason a 10 per cent decrease or increase in the peripheral resistance in the muscle vessels was considered to be the threshold vasodilator and vasoconstrictor response. The peripheral resistance (PR) was calculated according to the formula of Green et al. (1944), PR-unit $= \dfrac{1 \text{ mm Hg}}{1 \text{ ml/min}}$. The smallest infused amount producing the threshold response was considered to be the threshold dose.

For an account of determinations of the muscle blood flow, arterial pressure, heart rate and contractile force of the heart, as well as of the relevant surgical techniques, reference is made to a previous paper in this issue (p. 255). In 7 experiments the contractile force of the heart was measured with the modified strain gauge arch (Rosén 1961 a), and in 2 experiments with the original type (Boniface et al. 1953).

Results

Infusion of adrenaline

Adrenaline augmented cardiac contractile force with a mean threshold dose of 0.26 μg/kg/min with standard deviation $\pm$ 0.12. The mean threshold dose for vasodilatation in skeletal muscle was 0.44 $\pm$ 0.20 μg/kg/min.

One of the experiments is illustrated in Fig. 1. The series was started by infusion of 0.14 μg/kg/min of adrenaline (not included in the figure). This dose
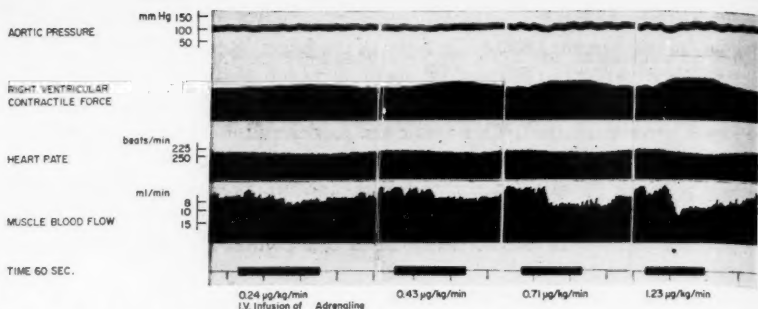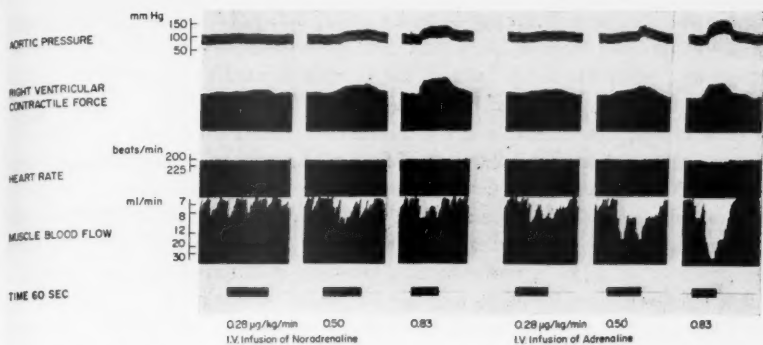
ANDERS ROSÉN

Fig. 1. Cat 3.5 kg. Aortic pressure, ventricular contractile force, heart rate and muscle blood flow responses to intravenous infusions of adrenaline.

produced no cardiovascular response. An increase in the contractile force of the right ventricle was, however, elicited by the next infusion (0.24 μg/kg/min), which was considered to be the threshold dose for an inotropic response. No vasodilator response appeared on infusion of this dose, whereas this was the case with 0.43 μg/kg/min, which was considered to be the threshold dose for a vasodilator response. In another illustrated experiment, the threshold doses were the same for the two responses, 0.28 μg/kg/min (Fig. 2). In all experiments, higher doses — up to the largest quantity infused, generally 1—2 μg/kg/min — still elicited vasodilatation in the muscles.

The heart rate was recorded in these two experiments, as in all the others. The response ranged from no increase in rate or a minimal one to a rise which, on infusion of quantities up to 1 μg/kg/min, seldom exceeded 10 per cent of the initial value.

Infusion of noradrenaline

In the 7 experiments with infusion of noradrenaline, no observations were made to indicate that any great quantitative difference is present between adrenaline and noradrenaline, as fas as their effect on the contractile force of the heart is concerned. The mean threshold dose of noradrenaline for a cardiac inotropic response amounted to 0.37 μg/kg/min with standard deviation $\pm$ 0.14. Noradrenaline caused muscle vasoconstriction with a mean threshold dose of 1.17 $\pm$ 0.45 μg/kg/min.

The effects of a series of noradrenaline infusions are shown in Fig. 2. The increase in the contractile force of the heart was of the same extent as that produced by the corresponding quantity of adrenaline. In this series, the threshold dose for a vasoconstrictor response was 0.83 μg/kg/min. No increase in heart rate was recorded, and in the remaining experiments that was in general also the case.

Fig. 2. Cat 3.0 kg. Aortic pressure, ventricular contractile force, heart rate and muscle blood flow responses to intravenous infusions of noradrenaline and adrenaline.

Discussion

The present investigation showed that intravenous infusion of adrenaline as well as of noradrenaline in a threshold quantity of 0.1—0.5 μg/kg/min produced an increase in the contractile force of the heart in the cat. It thus confirmed the observations of COTTEN and PINCUS (1955) and WEST and RUSHMER (1957), among others, that no great quantitative difference is present between the cardiac inotropic effect of adrenaline and noradrenaline. The literature contains very few quantitative data on the effect of intravenously infused catechol amines on the contractile force of the heart. WEST and RUSHMER found that intravenous infusion of 0.001 μM/kg/min of both adrenaline and noradrenaline produced an increase in the stroke work of the ventricle in the dog. That value, however, can not be compared with those, obtained in the present investigation. I found that infusions of adrenaline elicited slight to moderate tachycardia. Infusions of noradrenaline, on the contrary, generally resulted in no change in heart rate. These findings are largely in agreement with those of WEST and RUSHMER in the dog.

On hypothalamic excitation of the vasodilator pathway by stimuli of moderate intensity (0.5—2.5 V), with frequency 70/sec, GRANT et al. (1958) found the mean value for the output of adrenaline from one adrenal to be 0.23 μg/kg/min. The corresponding figure for noradrenaline was 0.30 μg/kg/min. On stimulation of the vasodilator pathway in the medulla oblongata with the same parameters, the discharge of adrenaline from one adrenal amounted to 0.20 μg/kg/min, and that of noradrenaline to 0.47 μg/kg/min (LINDGREN et al. 1959 a). If these figures are doubled to obtain the value for both adrenals, the excretion of catechol amines on the performed stimulations of the vasodilator outflow seems — on comparison with the threshold doses for cardiac inotropic responses re-

corded in the present study — to be amply sufficient to induce an increase in the contractile force of the heart. This observation may explain why adrenalectomy often markedly reduces the increased cardiac responses to central activation of the vasodilator outflow (ROSÉN 1961 a, b).

In addition, vasodilatation in the muscles was found to be produced by adrenaline in the mean threshold dose of 0.44 μg/kg/min with standard deviation $\pm$ 0.20. Noradrenaline, on the other hand, was unable to elicit vasoconstriction in the muscles until the dose reached 1.17 $\pm$ 0.45 μg/kg/min. These threshold doses are essentially in agreement with earlier observations (CELANDER 1954, LINDGREN et al. 1959 b, and others). LINDGREN et al. found that the quantity of adrenaline and noradrenaline released by hypothalamic and medullary stimuli of moderate intensity applied to the sympathetic vasodilator pathway was, in fact, too low to exert any appreciable vasomotor action. My observations have also led to this conclusion.

Using a paired t-test the p-value for the difference between the threshold dose of adrenaline, as well as of noradrenaline, for a cardiac inotropic and a muscle vasomotor response was < 0.01 but > 0.001. The question of whether this small difference is physiologically significant cannot be definitely answered. The two responses are indeed dissimilar, that is, the contractile force measurement represents a more direct parameter than does peripheral resistance, and furthermore, one cannot have confidence in quite comparable local tissue concentrations.

On electrical stimulation of the sympathetic vasodilator system, the quantity of catechol amines liberated from the adrenals has a greater effect on the contractile force of the heart than on the vasomotor tonus of the muscles. This is apparently due to the low threshold dose of adrenaline and noradrenaline required for a cardiac inotropic response.

The conceivable physiologic significance of the vasodilator system has been discussed in earlier publications (ELIASSON et al. 1951, UVNÄS 1954, ABRAHAMS et al. 1960, UVNÄS 1960, ROSÉN 1961 a, b). Here, it suffices to recall the possible implication in situations of emotional stress (ABRAHAMS et al. 1960), and in the initial stage of muscular work.

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The Effect of Acetylcholine and Related Substances on the Isolated Muscle Spindle

By

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Abstract

OTTOSON, D. *The effect of acetylcholine and related substances on the isolated muscle spindle.* Acta physiol. scand. 1961. *53.* 276—287. — In studies on the action of various drugs on the isolated frog's muscle spindle it was found that ACh in concentrations below 1×10^{-4} had no significant effect on the sensory endings of the spindle while higher concentrations $(5 \times 10^{-4}$—$1 \times 10^{-3})$ caused a slight reduction of their activity. Other cholinesters like butyrylcholine, propionylcholine and succinylcholine had a similar action as ACh. Nor-acetylcholine in concentrations of 5×10^{-5}—1×10^{-4} caused a suppression of the spontaneous activity and a reduction of the discharge during stretch. Of the anticholinesterases that were studied eserine, DFP and mintacol had an excitatory action that was followed by a cessation of the spontaneous discharge and a block of the response to stretch. Eserine was active in concentrations of 2×10^{-4}—1×10^{-3}, DFP in concentrations of 3×10^{-4}—1×10^{-3} and mintacol in concentrations of 5×10^{-6}—1×10^{-5}. Prostigmine had no significant action on the spindle. Histochemical determination of the cholinesterase activity in the spindle showed the presence of slight concentrations of cholinesterases in the polar regions of the spindle.

As shown by KATZ (1950) stretch sets up in the muscle spindle a local potential which secondarily gives rise to a discharge in the sensory axon. The development of the receptor potential can not be explained as due to an increase in the capacity of the membrane although such a mechanism may be involved in the production of the dynamic component of the response (KATZ 1950). The suggestion has therefore been made that the deformation of the sensory membrane causes permeability changes with a subsequent ionic flow. These

276

changes can be assumed to arise either as a direct result of the mechanical distortion of the membrane or they may be mediated through the release of some chemical transmittor (KATZ 1961). This last alternative raises the question of whether or not ACh is involved in the initiation of the receptor response.

The present paper describes some of the results of a study on the action of ACh and related substances on the activity of the isolated muscle spindle of the frog. One reason for using the isolated spindle was that in this preparation the test substances can be applied directly to the sensory endings. Another advantage lies in the fact that indirect effects which may confuse the interpretation of the results obtained in non-isolated preparations are absent or can easily be eliminated in the isolated spindle. Furthermore, since the receptor potential can be recorded together with the impulse discharge, it is possible to get clues as to the site of action of an applied substance.

The results obtained show that none of the quaternary ammonium compounds that were tested produced excitation whereas some anticholinesterases gave rise to an intense discharge from the spindle.

Methods

Preparation. The experiments were made on isolated spindles of *m. ext. dig. long. IV* of the frog. This muscle contains 2—3 spindle systems each of which consists of a bundle of intrafusal muscle fibres with the spindles arranged in series (GRAY 1957). The anatomical arrangement of these systems is usually very constant and the spindles are therefore easy to find. The dissection was carried out under dark field illumination with the muscle mounted in a Ringer bath. The first step was to locate a spindle and then to isolate the nerve bundle running to it. This bundle contains besides the sensory axon a number of motor fibres to the intrafusal muscles. The motor fibres sometimes follow the sensory axon to its entrance through the spindle capsule and from here they run along the spindle to the inter-sensory portions of the intrafusal muscles. In other cases the motor fibres leave the sensory axon at some distance from the spindle. All the fibres except the sensory axon were severed and the spindle cleared from adjacent tissue. The intrafusal bundle was then cut close to the ends of the spindle and the preparation lifted over into a smaller chamber (0.5×2 mm) where it was clamped at its ends to thin nylon rods (0.15 mm diam.) One of these rods was fixed to the wall of the chamber while the other was connected to an electromagnet by which stretches could be applied. The electromagnet was mounted on to a micromanipulator so that the resting length of the spindle could easily be changed.

It was often observed that tne intrafusal fibres in the mounted preparation showed slow tonic contractions or fast fibrillating twitches which produced a discharge from the spindle. This occurred most frequently when the intrafusal bundle had been cut at some distance from the spindle. When testing the effect of various compounds on the sensory endings it was essential to eliminate secondary effects caused by the action of the drugs on the intrafusal muscles. These were therefore always killed by pinching them as close as possible to the ends of the spindle. In addition it was sometimes found necessary to puncture the intracapsular portions of the intrafusal fibres with a coarse ($2—5\ \mu$) glass-capillary.

Recording. Recordings were made with Ag—AgCl electrodes connected to the preparation with glass tubes filled with Ringer-Agar. One electrode was placed in the Ringer
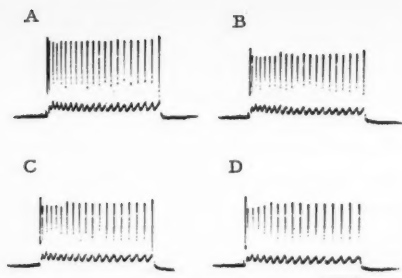
Fig. 1. The effect of acetylcholine on the stretch response. A, in Ringer. B—D, 5, 10, 30 min after application of ACh (1×10^{-3}). Time 100 msec.

solution in the chamber while the other was applied to the sensory axon, which was lifted up in oil. The Ringer-oil interface was at the portion of the sensory axon where it enters the capsule. The electrodes were connected to a direct coupled amplifier.

Electrical artifacts which were difficult to distinguish from the receptor potential were sometimes recorded when the spindle was stretched. Tests showed that these artifacts could not be explained as caused by the grid current $(1 \times 10^{-10}$ A) solely. In many cases they could be traced back to the presence of a polarization voltage at the recording electrodes. Therefore electrodes to be used were always first tested on a killed preparation to ensure that they gave no such artifacts.

Solutions. The following drugs were tested: acetylcholine chloride, butyrylcholine iodide, propionylcholine chloride, nor-acetylcholine (β-acetoxy-ethyl-dimethyl-dodecyl ammonium chloride), prostigmine, eserine salicylate, di-iso-propylfluoro phosphonate (DFP), p-nitrophenyl-diethylphosphate (mintacol), suxamethonium iodide, tubocurarine chloride and hexamethonium bromide. The solutions were always freshly prepared for each experiment. The concentrations are expressed in g/ml. The Ringer solution containing the drug to be tested was applied to the spindle after the fluid in the chamber had been partly removed with a pipette. Removal of all the fluid in the bath was not practical since this resulted in a positional change of the spindle and a discharge. Great care had therefore always to be taken so that the change of solution did not stretch the spindle. In order to replace all of the Ringer solution in the chamber with the test solution several changes of fluid had to be performed. This could usually be done in less than one min. To ensure that during this procedure no initial effects were overlooked all drugs were also tested by adding a small drop of a solution of high concentration to the bath so that the drug may reach the spindle by diffusion.

Results

When the spindle is kept at resting length it usually fires at a low rate. This discharge goes on for hours with no appreciable change in frequency and seems to be a sensitive index of the functional state of the spindle. It was often observed that drugs in concentrations which had no significant effect on the response to stretch produced a change in the spontaneous discharge rate, thus revealing that they were not entirely without effect.

The test stretches used in the present study were usually adjusted so as to give a moderate response. As a rule this response remained constant for periods
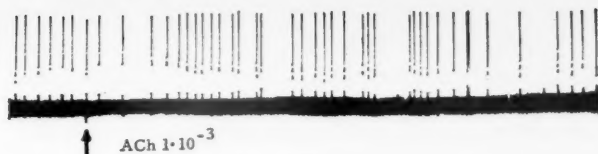
ACh 1·10^{-3}

Fig. 2. The absence of effect of acetylcholine on the spontaneous activity of the muscle spindle.

of several hours provided the spindle was not stimulated too frequently. However, within the first 10—15 min after the spindle was mounted the response to a given stretch often varied a great deal. The spindle was therefore always left to rest for about 15 min after being mounted, and no tests of the effects of drugs were done before the response had been constant for at least 15 min.

The effect of cholinesters

ACh in concentrations below 1×10^{-4} generally had no conspicuous action on the spindle. In concentrations of 5×10^{-4}—1×10^{-3} the drug caused a decrease in frequency of the spontaneous discharge. This change was usually very slight, but in some preparations the spindle became silent after having been soaked in the test solution for a few minutes. In concentration of 1×10^{-3} ACh usually also caused a reduction of the stretch response. This change took place slowly and did not become marked until the spindle had been exposed to the drug for 20—30 min. In many preparations which appeared to be in perfect condition ACh had very little effect, as is illustrated in Fig. 1. It must be emphasized that ACh in concentrations of 1×10^{-7}—1×10^{-3} was never observed to produce any initial excitatory action (Fig. 2). This lack of effect could not be due to an inability of ACh to pass through the spindle capsule, since identical results were obtained after the spindle capsule was split.

It is well known that ACh even in high concentrations is inactive on myelinated nerve fibres. The absence of effect has been explained as being due to the failure of ACh to reach the inside of the membrane (NACHMANSOHN 1959). Recently a number of lipid soluble quaternary ammonium compounds have been developed, which are reported to depolarize myelinated nerve fibre (STÄMPFLI 1958, DETTBARN 1959). One of these, referred to as nor-ACh, was tested on the spindle during the present study. It was found that this compound in concentrations of 5×10^{-5}—1×10^{-4} caused a pronounced decrease in the spontaneous activity of the spindle. This effect took place within a few seconds after application of the drug and often led to a cessation of the spontaneous discharge within 1—5 min. If the solution in the bath was not stirred or renewed, it sometimes occurred that the spindle after having been silent for some minutes started to fire again at a slow rate. Renewal or stirring of the solution immediately stopped this discharge. The response to
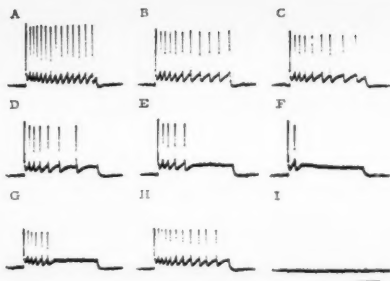
Fig. 3. The effect of nor-acetylcholine. A, in Ringer. B—F after 1, 5, 6, 10 and 12 min in nor-ACh (3×10^{-4} w/v). G—H, recovery after 10 and 30 min in Ringer. I, after the spindle had been pinched. Time 100 msec.

stretch also became reduced although this effect developed less rapidly than the decrease of the spontaneous discharge. Thus, stretches produced responses long after the spindle had ceased to fire spontaneously. As illustrated in Fig. 3 the effect of nor-ACh on the stretch response appears as a progressive decrease in the discharge until only a few impulses are left. The remaining impulses are relatively resistant to the action of the drug but finally drop out after prolonged exposure. It may be noted that the action of the drug on the sensory endings takes place more slowly than that on the axon as is revealed by the fact that the receptor potential remains almost unchanged at a stage when most of the nerve impulses are extinguished (record F). If the preparation was rinsed in Ringer before the response had disappeared entirely the activity of the spindle was partly restored (records G and H). Nor-acetylcholine was also tested together with subthreshold concentrations of eserine ($1—2 \times 10^{-4}$). The effect obtained was essentially the same as with nor-acetylcholine alone.

Of the other cholinesters that were tested butyrylcholine and propionylcholine had a similar action as ACh but were slightly less potent. Neither of them produced any excitatory effect. It has been demonstrated that succinylcholine has a stimulating action on non-isolated spindles (GRANIT et al. 1953, HENATSCH and SCHULTE 1958). In the isolated spindle of the frog a similar effect could not be obtained. In concentrations below 1×10^{-5} succinylcholine did not affect the spindle whereas higher concentrations ($2—5 \times 10^{-4}$) produced a slight reduction of the spontaneous discharge as well as of the response to stretch.

Anticholinesterases

The anticholinesterases studied in the present investigation were prostigmine, eserine, DFP and mintacol.

No readily detectable change in the activity of the spindle could be obtained with prostigmine in concentrations as high as $5 \times 10^{-5}—5 \times 10^{-4}$ (Fig. 4). In some preparations a decrease in the spontaneous activity occurred and the response to stretch became slightly reduced. These effects appeared slowly
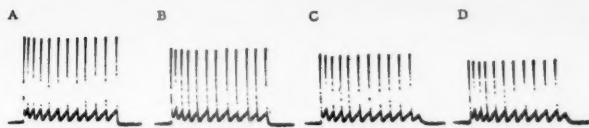
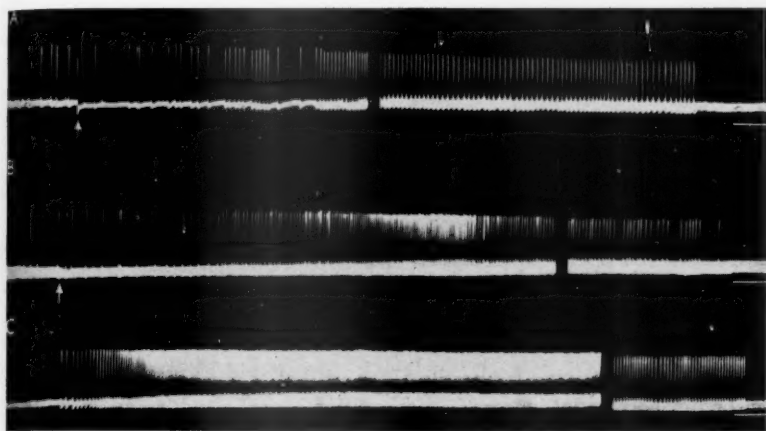Fig. 4. The effect of prostigmine. A, in Ringer. B—D after 1, 5 and 15 min in prostigmine (1 × × 10⁻⁴ w/v). Time 100 msec.

Fig. 5 A. The effect of eserine (5 × 10⁻⁴ w/v). Application of one drop of the solution (arrow) causes a discharge lasting for about 30 sec and ending abruptly. Time 1 sec.
B. The effect of DFP. One small drop of DFP (5 × 10⁻⁴) applied in the bath (arrow). Time 1 sec.
C. The effect of mintacol. One small drop of mintacol (1 × 10⁻⁵ w/v) applied in the bath about 5 sec before the recording was started. Note negative d. c. shift with increasing firing frequency. Time 1 sec.

and did often not become marked until after long (30—45 min) exposures. It could therefore not be ascertained whether or not these changes could be ascribed to the action of the drug.

Eserine in concentrations of 2×10^{-4}—5×10^{-4} usually produced an initial excitatory effect (Fig. 5 A) followed by a subsequent reduction of the sensitivity of the spindle to stretch and finally a complete block. The excitatory effect varied a great deal from one spindle to another. In some preparations there occurred a vigorous discharge that started 1—3 sec after application of the drug and usually lasted for 20—40 sec. Most often the firing ceased suddenly and the spindle thereafter remained silent. As with nor-ACh a certain recovery sometimes occurred if the solution was not stirred or renewed (cf. EDWARDS and KUFFLER 1959). In other preparations eserine only caused an inconspicuous

Fig. 6. The effect of eserine. A, in Ringer. B—G after 1, 3, 4, 5, 6 and 10 min in eserine (5×10^{-4} w/v). H—I, 1 and 3 min after washing with Ringer.

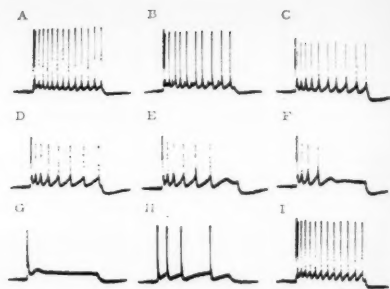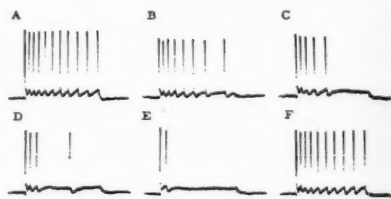Fig. 7. The effect of DFP on the stretch response. A, in Ringer. B—E after 1, 2, 3 and 4 min in DFP (2×10^{-4} w/v). Record F, 2 min after washing with Ringer.

increase of the activity. It was also observed that application of the compound in high concentrations (1×10^{-3}) sometimes caused a sudden cessation of the spontaneous activity without any preceding period of increased firing.

The response to stretch was increased during the period of intense firing, whereas after the spindle had become silent a rapid decrease occurred. As seen in record B—G in Fig. 6, the discharge during stretch was diminished in a way reminiscent of the effect produced by nor-ACh. It may be noted that in the early stage of the block there appear abortive spikes (B) and non-propagated prepotentials (E and F). As the impulses gradually drop out, the receptor potential becomes uncovered and is finally obtained almost isolated (G). The blocking effect on the discharge is reversible, and the original activity is restored within a few minutes after the preparation is rinsed in Ringer (I).

In concentrations of 3×10^{-4}—1×10^{-3} DFP produced an intense discharge that began a few sec after the test solution was applied. As seen in Fig. 5 B, there occurs at the same time a negative d. c. shift, indicating a depolarization of the spindle. The excitatory effect usually lasted not more than 10—20 sec, and most often the discharge stopped abruptly. The ensuing reduction of the excitability took place considerably faster than with eserine and sometimes led to a complete abolition of the impulse discharge within a few sec. Stretches applied immediately after the discharge had stopped often gave only one or a few impulses superimposed on a barely detectable local potential. With threshold concentrations the blocking effect took place less rapidly and the de-
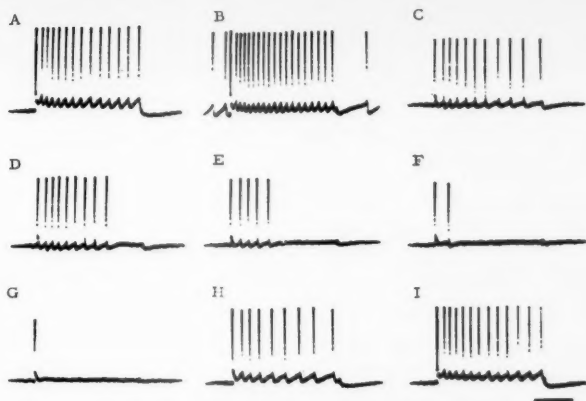
Fig. 8. The effect of mintacol on the stretch response. A, in Ringer. B—G, after 1/2, 1, 1 1/2, 2, 3 and 5 min in mintacol (5×10^{-6} w/v). Record B was obtained during the period of intense firing, C—G after the spindle had become silent. Records H—I, 2 and 3 min after washing with Ringer. Time 100 msec.

velopment of the block could be studied. As seen in Fig. 7, the effect is similar to that caused by eserine. The impulse discharge is again more sensitive to the action of the drug with the result that the receptor potential is left partly uncovered. However, this potential also undergoes a rapid reduction and is abolished if the preparation is exposed to the drug for some minutes. In the experiment illustrated in Fig. 7 the preparation was washed in Ringer after record E was obtained. As seen in record F, obtained 2 min later, the spindle regains its excitability rapidly.

Mintacol had an effect much like that of eserine and DFP. In threshold concentrations of 3×10^{-6}—5×10^{-6} the compound caused an increase in the spontaneous activity and a sensitization of the spindle to stretch. This effect usually vanished within a few minutes but could sometimes be maintained for 10—15 min by repeated changes of the solution in the bath. In higher concentration (1×10^{-5}) mintacol produced a vigorous discharge lasting for 30—40 sec (Fig. 5 C). The sensitivity to stretch was increased during this period as is illustrated by record B (Fig. 8). After the spindle had ceased to fire there followed a fast reduction of the response (C—G). It may be noted that this change also involved a rapid diminution of the receptor potential. The effect caused by mintacol was usually reversible, and the activity was rapidly restored if the spindle was rinsed in Ringer (record H—I Fig. 8). It was a constant observation that a second application of the drug shortly after the activity had been restored with a rinse of Ringer failed to produce any effect or only gave rise to a short burst of impulses. Only if the concentration was raised could the earlier
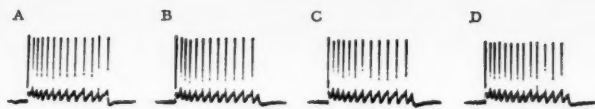
Fig. 9. The effect of tubocurarine. A, in Ringer. B—D after 10, 20 and 30 min in tubocurarine $(3 \times 10^{-4}$ w/v). Time 100 msec.

excitatory effect be obtained again. The same finding was made with DFP. The excitatory effect of eserine, DFP and mintacol was not obtained after the spindle had been pinched.

Blocking agents

Tubocurarine in concentrations of 1×10^{-6}—1×10^{-4} as a rule failed to produce any significant effect. When the concentration was raised (3×10^{-4}) a slight reduction of the response and of the spike height was sometimes obtained (Fig. 9). However, this effect often did not become detectable until after comparatively long exposures. Hexamethonium in concentration of 1×10^{-4} had an action similar to that of curare.

Cholinesterase-staining of the spindle

In connection with the study of the action of the different compounds described above a number of spindles were also stained according to the method developed by HOLMSTEDT (1957). It was found that no staining occurred in many of the preparations while others showed a slight positive AChE reaction. When staining occurred it was always confined to the polar regions of the spindle (Fig. 10). This finding is consistent with earlier observations (COËRS and DU-RAND 1956, GIACOBINI and HOLMSTEDT 1960).
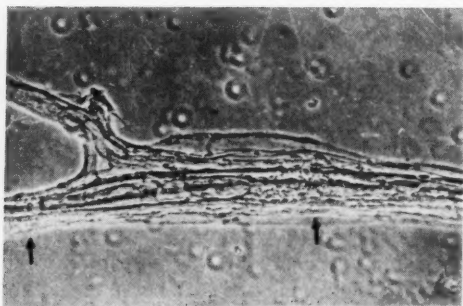

Fig. 10. Cholinesterase staining of isolated spindle. Note slight positive reaction at polar ends (arrows).

Discussion

As was first shown by CAJAL in 1888 the sensory fibre of the muscle spindle breaks up into fine unmyelinated strands with numerous varicosities distributed along the intra-capsular portion of the intrafusal fibres. According to KATZ (1961), who quite recently has published a report on the electron-microscopic structure of the frog's spindle, these varicosities consist of bulbous expansions which are 2—3 μ thick and connected through thin (0.15 μ in diam.) tubes. There are reasons to believe (KATZ 1961) that these bulbs form the mechano-electrical transducers in the spindle system. The first step in the electrical event leading to the sensory discharge must accordingly occur in the membrane of these structures. It is not possible to decide at present whether or not this first step involves the release of some active substance. It has been found in studies on curarized frog's whole muscles that ACh excites the spindle (HENATSCH and SCHULTE, 1958). In the cat the spindle response to intravenous injection of ACh is blocked by prior administration of tubocurarine (HUNT, 1952). No excitatory action was obtained in the present study when ACh was applied directly on the isolated spindle. The lack of effect can not be explained by the assumption that the spindle capsule offers a diffusional barrier since splitting of the capsule did not alter the experimental result. However, the failure of ACh to excite the isolated spindle can not be considered as a conclusive evidence against the ACh-theory since the receptor membrane may be impermeable to the compound. On the other hand the fact that nor-ACh, which is supposed to penetrate the membrane, also failed to excite the spindle seems to make it unlikely that ACh or any related substance is involved in the excitatory process.

In striking contrast to the relative ineffectiveness of the cholinesters stands the powerful action of the anticholinesterases. With the exception of prostigmine they all had essentially the same action: an initial excitatory effect and a subsequent reduction of the activity of the spindle. The fact that prostigmine did not cause these effects is most likely to be ascribed to the failure of the compound to penetrate lipid membranes. It has been demonstrated that prostigmine in concentrations as high as 0.01 M leaves conduction in nerves unaffected (BULLOCK et al. 1946). Eserine on the other hand is known to pass readily through the nerve membrane. It blocks conduction when applied externally to nerves (BULLOCK et al. 1946, LORENTE DE NÓ 1947, DETTBARN 1959, WRIGHT 1956). This effect is not due to a depolarization, since eserine does not reduce the resting potential of the nerve (LORENTE DE NÓ 1944, STRAUB 1955). DFP gave an effect very much like that of eserine but was more potent as judged from the finding that it caused a more rapid and intense effect. This can most likely be ascribed to the fact that DFP is more lipid than water soluble.

The excitatory effect of eserine, DFP and mintacol raises the question as to the mode of action of these substances. The main argument against the idea that they excite the spindle by blocking the cholinesterase activity is the finding that

neither ACh nor any other of the quaternary ammonium compounds that were
tested produced excitation. Furthermore the finding that the spindle contains no
or only very small amounts of cholinesterase suggests that the excitatory effect
of the anticholinesterases may not be mediated by the ACh system. This view
is also supported by the observation that eserine and DFP had to be used in
relatively high concentrations. It has been demonstrated that anticholinesterases
also may affect other enzyme systems (MICHAELIS *et al.* 1949). The possibility
therefore exists that the effect of eserine and DFP on the spindle is unspecific.
The fact that mintacol is active in concentrations as low as 3×10^{-6} suggests,
however, that this compound may have a specific action. Whatever the ex-
planation may be as to the precise mode of action of the inhibitors, the findings
suggest the existence of an enzymatic system involved in the processes main-
taining the membrane potential of the sensory endings.

The effect of anticholinesterases has been studied in many different types of
sensory end organs. In most of them no effect has been found while in a few
types an increased activity has been demonstrated. Thus, it has been shown
that eserine augments the activity of the chemoreceptors of the cats carotid
sinus (LANDGREN *et al.* 1952). It has also been demonstrated that prostigmine
increases the activity of the taste fibres in the cat (LANDGREN *et al.* 1954). One
may ask why these compounds do not have similar effects on other types of
sensory end organs. The explanation to this seems to be that the inhibitors have
to reach the active sites in sufficiently high concentrations within a limited dura-
tion of time. Sensory endings which are surrounded by tissues acting as dif-
fusional barriers will only be gradually reached by an externally applied drug.
The probability that the excitatory effect will be obtained is therefore greatly
increased in isolated preparations or in preparations where the receptors are
easily reached by the drug.

It has been pointed out that there is very little likelyhood that ACh participates
in the generation of the response of sense organs to their physiological stimuli
(GRAY and DIAMOND 1957). The results obtained in the present study indicate
that this may also be true for the spindle although the effect of the anticholin-
esterases suggests the possibility that chemical processes may be involved in
the initiation of the discharge.

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So-called Free Erythrocyte Protoporphyrin and its Possible Role in Hemoglobin Formation

By

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Abstract

ERIKSEN, L. *Socalled free erythrocyte protoporphyrin and its possible role in hemoglobin formation.* Acta physiol. scand. 1961. *53.* 288—299. — The relations of so-called free erythrocyte protoporphyrin to the number of reticulocytes in the blood of rabbits made anemic by daily injections of 25 mg phenylhydrazine for 5 days, has been studied. The heme forming capacity of the red cells as related to the number of reticulocytes has also been studied. All the data obtained are compatible with the assumption that the amount of free protoporphyrin is not strictly related to the number of the reticulocytes but to the stage of maturation of these cells. The possible role of free erythrocyte protoporphyrin in hemoglobin formation has also been studied, and it has been shown that the main pool of protoporphyrin is not normally utilized for heme formation. It is suggested that free protoporphyrin, although probably a normal intermediate in heme formation, when formed in excess diffuses out into the soluble part of cytoplasm and becomes unavailable for heme formation. The possibility that the true proto-type intermediate in heme formation differs chemically from protoporphyrin IX is, however, not excluded. It is suggested that excess formed protoporphyrin may in part be the origin of the so-called early bile pigment fraction found in mammals.

It has long been known that the erythrocytes in the peripheral blood of mammals do contain small amounts of free protoporphyrin (VAN DEN BERG, GROTEPASS and REEVERS 1932). SEGGEL (1940) was able to show that the porphyrin was present in the cells as a red fluorescing compound with a fluorescence spectrum identical with that of protoporphyrin. SEGGEL also presented evidence that the number of protoporphyrin containing cells, the so-called fluorocytes, increased in anemia and conditions characterized by an increased

inflow of young red cells into the peripheral blood. WATSON and CLARK (1937) first assumed that the protoporphyrin containing cells were the reticulocytes. However, WATSON later partly withdrew this assumption suggesting that the fluorocytes although young cells, were not identical with reticulocytes (WATSON, GRINSTEIN and HAWKINSON 1944). SCHWARTZ and WIKOFF (1952) in keeping with the later results of Watson and co-workers found no correlation between the amount of free protoporphyrin and the number of reticulocytes in the peripheral blood. However, neither WATSON et al. nor SCHWARTZ and WIKOFF did control that the population of reticulocytes studied actually could be compared, and since it has been shown by a series of authors that the reticulocytes may exist in the blood up to 4 days (for reference see SEIP 1953), it might very well be that the age of the reticulocyte may be the factor of importance in these studies and not the actual number of reticulocytes.

The possible role of the free erythrocyte protoporphyrin has long been debated, and it has become more and more probable that it is in some way directly related to hemoglobin formation. GRANICK (1954) presented indirect evidence that protoporphyrin added to or accumulated in young erythrocytes can be utilized for heme formation. Since then a series of authors (ERIKSEN 1956, KREUGER, MELNICK and KLEIN 1956, MINAKAMI, YONEYAMA and YOSHIKAWA 1958, NISHIDA and LABBE 1959) have shown that added protoporphyrin is readily utilized for heme formation in a series of different systems. However, DRESEL and FALK (1956) found that unlabelled protoporphyrin added to chicken hemolysates was unable to dilute the incorporation of ^{14}C into heme from different protoporphyrin precursors. They concluded that protoporphyrin similar to uroporphyrin and coproporphyrin does not lie on the pathway to heme but is a side product.

Since protoporphyrin is relatively insoluble at the pH used by DRESEL and FALK, their finding might be due to imcomplete mixing of the protoporphyrin added and that formed by the hemolysate. However, in view of our own finding that the non-heme protoporphyrin of rabbit reticulocytes was present in the cells in at least two metabolically different fractions (ERIKSEN 1955 a, 1956), the possibility arose that the two protoporphyrin fractions found were not, as interpreted at the time, two steps in the normal formation of heme, but that only one was an intermediate and that the other was a side product differing from the true intermediate either in its chemical properties or in its localization within the cell or both.

The present investigation was undertaken to get more information on the so-called free erythrocyte protoporphyrin and its role in hemoglobin formation.

Material and methods

In the in vivo experiments 1-year-old rabbits of the laboratory stock were used.

In all in vitro experiments blood with reticulocyte numbers of 60—70 per cent were obtained from rabbits made anemic by daily subcutaneous injections of 25 mg phenylhydrazine for 5 days and bled to death on the 7th day. Cells and plasma were separated

by centrifugation for 15 min at a speed of 2,000 r. p. m. The cells were washed once with 0.9 % NaCl and finally suspended in two volumes of 0.9 % NaCl. This suspension was used as a stock suspension in all the incubation experiments described below except where otherwise has been stated.

As tracer substances were used acetate labelled in the carboxyl group (1 mc/mole) or the methyl group (10 mc/mmole) with ^{14}C, and ferric chloride labelled with ^{55}Fe (50 mc/g Fe). The radioactive substances were obtained from Atomic Centre, Amersham, England.

Protoporphyrin and hemin were extracted from the reticulocytes with ethyl acetate and glacial acetic acid 4 : 1. The free porphyrins were brought out with 3 N HCl, fractionated and purified in the usual way (ERIKSEN 1956). The amount of free protoporphyrin was estimated according to GRINSTEIN and WINTROBE (1948). The identity of protoporphyrin was established by means of spectrophotometry and paper chromatography (ERIKSEN 1953). The specific activity of free protoporphyrin was measured as described elsewhere (ERIKSEN 1956).

Hemin was brought out of the ethyl acetate extract with 10 % ammonia after complete removal of all free porphyrins with 3 N HCl. The hemin was precipitated by acidification with small amounts of concentrated HCl, washed with 2 N HCl and distilled water, purified, plated and counted as described in detail elsewhere (ERIKSEN 1955 b, 1956).

In some experiments in which ^{55}Fe was used as tracer for the labelling of heme, the hemin prepared as described above was digested and the iron plated electrolytically and counted as described elsewhere (ERIKSEN 1955 b, 1956).

The reticulocyte counts were not performed directly on smears stained with brilliant cresyl blue, but after washing with methanol and restaining with diluted Giemsa solution. This was done since it was found that the direct technique gave too high values in phenylhydrazine poisoning (ERIKSEN and JACOBSEN 1960).

Experimental and results

The relation of free protoporphyrin to the number of reticulocytes: 8 rabbits were injected subcutaneously with 25 mg phenylhydrazine for 5 days. The number of reticulocytes and the amount of free protoporphyrin were measured each day at 10 o'clock in the morning prior to a new injection. Since SCHWARTZ and WIKOFF (1952) found no correlation between the number of reticulocytes and the amount of free protoporphyrin, but a correlation factor of approximately 1 for coproporphyrin and the number of reticulocytes, we also measured the amount of the 0.1 N HCl extractable porphyrin which was present in the cells in small amounts. This porphyrin was not tested for purity by paper chromatography, however, the porphyrin was insoluble in chloroform, was readily brought out of ether with 0.1 N HCl, and the spectral absorption in the Soret band was identical with coproporphyrin, so there is good reason to believe that we actually are dealing with coproporphyrin.

As seen from Fig. 1, there is a good correlation between the number of reticulocytes and both protoporphyrin and coproporphyrin, with a maximum for all of them on the 7th day. However the increase in the number of reticulocytes from the 6th to the 7th day is far less than the increase in free protoporphyrin and coproporphyrin.
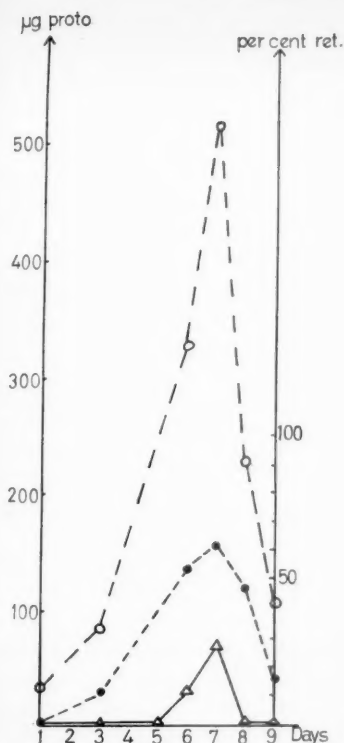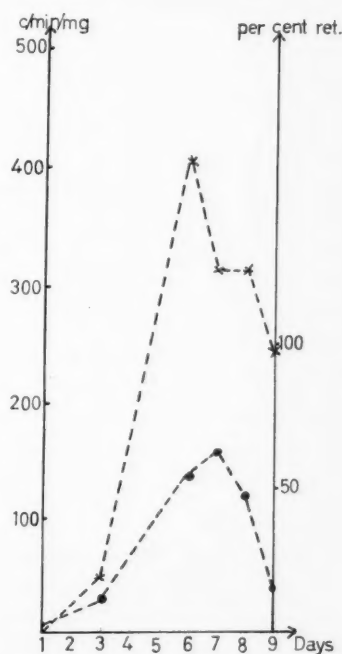
Fig. 1. The relation of free erythrocyte protoporphyrin and coproporphyrin to the number of reticulocytes in the blood of rabbits made anemic by daily injections of 25 mg phenylhydrazine for 5 days.

•- - -• per cent reticulocytes, ○— — —○ μg protoporphyrin/100 ml cells,
△———△ μg coproporphyrin/100 ml cells.

Fig. 2. Heme forming capacity of red cells from anemic rabbits as related to the number of reticulocytes. The red cells were suspended in 2 vols. 0.9 % NaCl/glycine (0.03 M) and incubated aerobically with ^{14}C-acetate as tracer.

×- - -× c/min/mg hemin, •- - -• per cent reticulocytes.

Heme-forming capacity of red cells from anemic rabbits as related to the number of reticulocytes: Rabbits no. 1 to 6 were killed on the 1st, 3rd, 6th, 7th, 8th and 9th day. The washed red cells were suspended in 0.9 % NaCl/glycine (0.03 M) and incubated in air for 1 hour with labelled acetate as tracer, and the specific activity of hemin was measured. As seen from Fig. 2, the maximum specific activity is reached on the 6th day, while the maximum of reticulocytes is reached on the 7th day. The relatively slow drop in the specific activity of hemin shows that the ability to synthesize heme from simple building stones
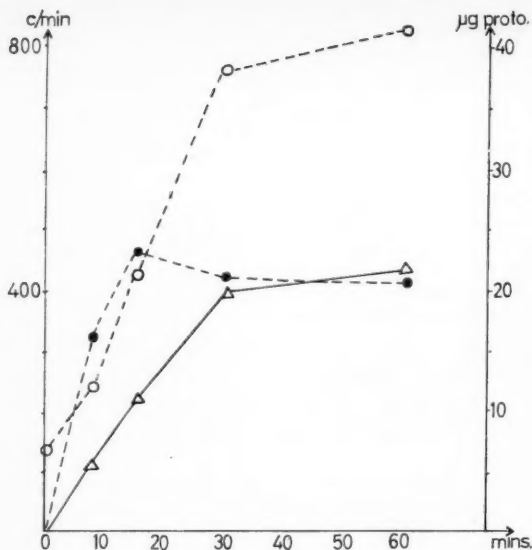
Fig. 3. The formation of free protoporphyrin and the incorporation of ^{14}C-acetate into protoporphyrin and heme by rabbit reticulocytes suspended in 2 vols. 0.9 % NaCl/glycine (0.03 M) and incubated in air.
o – – –o µg protoporphyrin,
•– – –• c/min/µg protoporphyrin,
△————△ c/min/mg he. min.

such as acetic acid is not specifically linked to cells having a "substancia granulofilamentosa", but is a property of the red cell up to a certain stage of maturation. So far, we have no morphological means to identify these cells, however, it might be that if the reticulocytes were classified according to HEILMEYER (1931) and correlated to the above parameters, one would be able to classify them according to their age and biochemical properties, and thus get some morphological reference for the biochemical and physiological properties of the red cells in their early phase of maturation. It seems quite clear from the above results that the number of reticulocytes could not be used as a reference neither for the amount of free protoporphyrin nor for the ability to synthesize heme.

The utilization of preformed protoporphyrin for heme formation in whole cell systems in vitro: The possible fate of the protoporphyrin accumulated in the young cells is so far unknown. It might be that it is a normal precursor to heme and is utilized for hemoglobin formation in the organism. Such an assumption is supported by the finding of LONDON, SHEMIN and RITTENBERG (1950) indicating that hemoglobin formation continues in the reticulocytes in the peripheral blood of the rabbit. Similar findings have been reported by REIZENSTEIN and OGATA (1957) in the rat.

However, since the reticulocytes are fully capable to synthesize both the porphyrin and the globin part of hemoglobin from such simple building stones
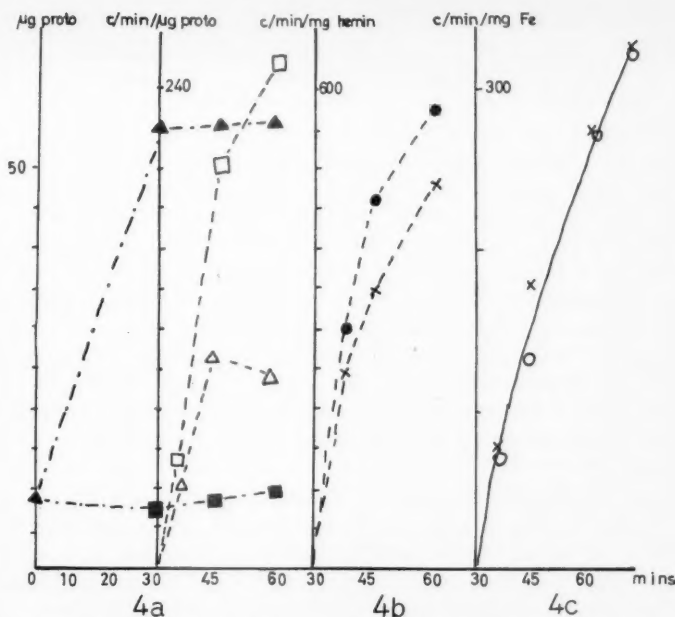
Fig. 4 a, b, c. The incorporation of ¹⁴C-acetate into protoporphyrin and heme and ⁵⁵Fe into heme by rabbit reticulocytes suspended in 2 vols. 0.9 % NaCl/glycine (0.03 M) and incubated in air. The cells had been preincubated aerobically in 0.9 % NaCl with and without addition of glycine for 30 min prior to the addition of the tracer substances.
Glycine series: ▲—·—▲ μg proto, △- - -△ c/min/μg proto, ●- - - -● c/min/mg ¹⁴C-hemin, ○——○ c/min/mg Fe.
Nonglycine series: ■—·—■ μg proto, □- - -□ c/min/μg proto, ×- - - -× c/min/mg ¹⁴C-hemin, ×——× c/min/mg Fe.

as acetic acid and the free amino acids (KRUH and BORSOOK 1956, ERIKSEN 1957, NIZET 1957, MORELL, SAVOIE and LONDON 1958), it might be that the protoporphyrin present in young red cells differs from that of the true intermediate in its localization or its chemical properties or both. If preformed protoporphyrin lay on the pathway to heme, one would expect that the specific activity of heme should follow very closely that of free protoporphyrin, and cells suspended in 0.9 % NaCl/glycine (0.03 N) containing 2 μc methyl labelled acetate per 5 ml of cells, were incubated for 60 min in air. Samples were taken after 0, 7, 15, 30 and 60 min, and the amount of free protoporphyrin and the specific activities of free protoporphyrin and hemin were measured.

As seen from Fig. 3, the amount of free protoporphyrin increases rapidly to approximately 5 times its zero value in 30 min, from then on the increase is only slight. The curves for the specific activities of free protoporphyrin and

19—613015. *Acta physiol. scand. Vol. 53.*

hemin are not consistent with the assumption that preformed protoporphyrin is metabolically equal to the newly formed true intermediate, but indicate that more than one pool of protoporphyrin (or protoporphyrin precursor converted into protoporphyrin during the extraction procedure) is present in the system, and that only one of these is utilized for heme formation.

To test this possibility 2 bottles containing 40 ml of the standard reticulocyte suspension were incubated in air. To bottle 1 glycine was added in optimal concentration for protoporphyrin formation (ERIKSEN 1956). After 30 min incubation the cells and media were separated by centrifugation and the cells suspended in 2 volumes of 0.9 % NaCl plus ferric chloride in optimal concentration for heme formation (ERIKSEN 1956). Four series of bottles, two of which contained the cells preincubated with glycine and two containing the cells from the non glycine medium, were further incubated. As tracers were used carboxyl labelled acetate and ^{55}Fe as ferric chloride. The incubation was stopped after 0, 7.5, 15 and 30 min, and the amounts of free protoporphyrin and the specific activities of free protoporphyrin, hemin and hemin iron were measured.

As seen from Fig. 4 a, the amount of free protoporphyrin in the cells preincubated with glycine is considerably higher than in the non glycine cells. In both series the amount of preformed protoporphyrin keeps approximately constant throughout the second incubation. Thus if preformed protoporphyrin has been utilized for heme formation, an equivalent amount of protoporphyrin must have been formed.

The amount of heme formed as judged from the amount of ^{55}Fe incorporated, is equal in both series (Fig. 4 c), and the specific activity of free protoporphyrin from the non glycine cells is approximately twice that of free protoporphyrin from cells preincubated with glycine. If preformed protoporphyrin was equally well utilized for heme formation as the newly formed true intermediate, the ^{14}C-activity of the hemin should vary in the same way as that of the free protoporphyrin. However, as seen from Fig. 4 b, the specific activities of the ^{14}C-labelled hemins are of the same order in both series and even slightly lower in the non glycine series. Thus the specific activity of free protoporphyrin could not be used as a measure of the specific activity of the true intermediate. It is further obvious that the true intermediate bypasses the main pool of protoporphyrin. Since, however, the specific activity of free protoporphyrin increases relatively rapid even in the glycine series and without any increase in the amount of free protoporphyrin, it means that a smaller part of the true intermediate is present and resynthesized during the second incubation.

Heme formation in hemolysates and whole cell systems under hypoxic conditions: The possibility that the only difference between the two pools is the localization within the heme-forming system, would fit better with our earlier finding that added ^{14}C-labelled protoporphyrin is readily utilized for heme formation by rabbit reticulocytes in vitro (ERIKSEN 1956).

Table I. Incorporation of ^{55}Fe and disappearance of preformed protoporphyrin in hemolysates incubated in air or a stream of N_2/CO_2

Time in min	Atmosphere	c/min/mg hemin Fe	per cent ^{55}Fe incorp.	Drop in proto	
				in μg	in per cent of aerobic drop
30	air	1,784	100	23.4	100
30	N_2/CO_2	2,212	123.9	28.8	122.6

To test this possibility, lysates of reticulocytes preincubated aerobically for 30 min in 0.9 % NaCl/glycine (0.03 M) were prepared by a method slightly different from that of Dresel and Falk (1954) and incubated in two series with ^{55}Fe as tracer. The only difference between our method and that of Dresel and Falk was that the cells were lysed at room temperature instead of at 0—4° C as used by these authors. A lysate prepared in this way has lost its ability to synthesize protoporphyrin and heme from acetate and glycine, but is still capable of forming heme from tetrapyrrolic intermediates (ERIKSEN, unpublished data).

Series 1 was incubated in air and series 2 was incubated in a stream of commercial N_2/CO_2 (95/5). The amount of free protoporphyrin and the specific activity of hemin iron were measured at zero time and after 30 min of incubation. As seen from Table I, more heme has been formed in the N_2/CO_2 series than in the aerobic series. In both series heme formation is followed by a drop in free protoporphyrin, and the drop is higher in the N_2/CO_2 series than in the aerobic series. The incorporation of iron and the drop in free protoporphyrin in the N_2/CO_2 series in per cent of the changes in the aerobic series are approximately identical indicating that the lost protoporphyrin has been utilized for heme formation. The results also support the above suggestion that the metabolic difference between the protoporphyrin fractions studied is due to the localization in the system used and not to chemical difference between protoporphyrin and the normal intermediate.

Since the N_2/CO_2 atmosphere seems to favour the insertion of iron into protoporphyrin, the possibility existed that heme formation from preformed protoporphyrin might take place even in whole cells in a similar atmosphere, and experiments designed to test this possibility were undertaken. We soon found that when true anaerobiosis was obtained, no or negligible amounts of heme were formed, while with a gassing procedure identical with that used in the above experiments, iron was utilized for heme formation even better than in air. However, the drop in protoporphyrin was small and far from equivalent to the amount of heme formed. Thus the heme formed could not be due to utilization of preformed protoporphyrin, but must originate from the newly synthesized intermediate.

Table II. *Incorporation of ^{55}Fe and ^{14}C-acetate into heme in rabbit reticulocytes suspended in 2 vol. 0.9 % NaCl/lycine and incubated in air or a stream of N_2/CO_2 for 2 hours*

Atmosphere	c/min/mg ^{14}C-hemin	c/min/mg hemin-^{55}Fe	sp. act. ^{55}Fe sp. act. hemin-^{14}C
air	100.0	18,460	184.6
N_2/CO_2	112.2	20,885	186.2

In Table II the results are given of an experiment designed to establish the possible origin of the newly formed heme.

Four series of cells suspended in 0.9 % NaCl/glycine were incubated for 2 hours. Series 1 and 2 were incubated in air, and series 3 and 4 were incubated in N_2/CO_2. As tracers were used ^{14}C-labelled acetate and ^{55}Fe as ferric chloride.

As seen from the table, the amount of heme formed as judged from the amount of iron incorporated is approximately 10 per cent higher in the N_2/CO_2 series as compared to the aerobic series, in good agreement with the findings in the experiments with hemolysates. However, the ^{14}C incorporation has increased to the same degree, and the ratio of ^{14}C-hemin/hemin-^{55}Fe is identical in both series. Thus the heme formed must have the same origin in both series.

The N_2/CO_2 mixture was found to contain less than 3 per cent oxygen, and the incubation bottles were fluxed with the two gas mixtures for 60 min prior to the introduction of tracer, thus the condition in the N_2/CO_2 series must be highly hypoxic, and even under these conditions the heme forming system seems to prefer the newly formed intermediate corresponding to protoporphyrin for preformed protoporphyrin.

Discussion

As shown in Fig. 1, the amount of both free protoporphyrin and coproporphyrin are closely related to the number of reticulocytes both during the development and the repair of a heavy anemia. However, the form of the curves is compatible with the assumption that the main part of the free porphyrins is present in cells of an intermediate maturation state, which would explain the findings of WATSON and CLARKE (1937), WATSON et al. (1944) and SCHWARTZ and WIKOFF (1952).

The rapid drop in free porphyrin from the 7th to the 9th day might be due either to utilization of the porphyrins for heme formation or disposal of them by other routes. Since as shown in Fig. 3 and 4 preformed protoporphyrin is only poorly utilized for heme formation in vitro although heme formation from simple metabolites such as acetic acid goes on with a considerable speed, and

since preformed protoporphyrin is incapable to dilute the incorporation of ^{14}C from added acetic acid, it seems reasonable to assume that the true intermediate corresponding to protoporphyrin differs from preformed protoporphyrin either in its site of localization or in its chemical properties or both.

Added protoporphyrin is readily utilized for heme formation both in whole cell systems (ERIKSEN 1956) and cell free systems (KEYGER et al 1956, GOLDBERG et al. 1956, MINAKAMI et al. 1958), thus if the true intermediate does differ chemically from protoporphyrin, the latter must be easily converted into the former even in such extremely simple systems as the 50 per cent ammonium sulfate fraction of cholate extracts of the insoluble part of duck erythrocytes described by KAGAWA, MINAKAMI and YONEYAMA (1958).

The observation that higher amounts of heme are formed when the incubation is performed in a stream of N_2/CO_2 (Table I and II, and GRANICK, 1954) and the findings of several authors that reducing substances such as cysteine, glutathione and ascorbic acid increase heme formation from protoporphyrin (MINAKAMI 1958, MINAKAMI et al. 1958, GOLDBERG 1959, NISHIDA and LABBE 1959), might suggest that some reduction of the protoporphyrin molecule is necessary for the utilization in heme formation. However, as shown by KAGAWA et al. (1958) these compounds seem to play no role as reductants neither for protoporphyrin nor for iron, but to protect some easily oxidizable group or compound in the heme forming system. It has also been shown by NISHIDA and LABBE (1959) that protoporphyrin is more readily utilized for heme formation than protoporphyrinogen in cell free systems.

Thus, although the question can not be said to be settled, the evidence is in favour of the assumption that protoporphyrin in contrast to other porphyrins is a true intermediate in heme formation, and that the non utilization of preformed protoporphyrin is due to the localization of excess formed protoporphyrin in the system studied, an assumption which is favoured by our own finding (Table I) that in disrupted cells preformed protoporphyrin is readily utilized for heme formation.

Since the cells used in the above in vitro experiments most probably are a population of cells of different maturation and thus with different ability to form porphyrins and heme, it becomes difficult to decide whether this "compartmentisation" of protoporphyrin can occur within a single cell or is due to the distribution of protoporphyrin between cells of different maturation. However, DRESEL and FALK's observation that protoporphyrin added to hemolysates does not dilute the activity incorporated into heme from different tracers added when the lysates are prepared in such a way that the heme forming enzymes are still located in the particulate matter (DRESEL and FALK 1956), indicates that this phenomenon does occur even in non cellular systems, and preliminary results dealt with elsewhere (ERIKSEN and ERIKSEN 1960) indicate that the formation and utilization of protoporphyrin for heme formation is connected with the particulate matter of the reticulocytes, and that excess formed proto-

porphyrin diffuses out into the soluble part of cytoplasm and becomes unavailable for heme formation.

The fate of this protoporphyrin fraction is so far unknown, but preliminary investigations suggest that the ultimate fate of excess formed protoporphyrin is the breakdown to bile pigment or related compounds, and the possibility exists that the socalled "early" bile pigment fraction formed in direct relation to hemoglobin biosynthesis in mammals (LONDON et al. 1950, GRAY, NEUBERGER and SNEATH 1950) does origin from this protoporphyrin fraction, and experiments designed to test this possibility are in progress at our institute.

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The Effect of Cobalt Ions on the Biosynthesis of
Hemoglobin by Rabbit Reticulocytes in Vitro

By

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Abstract

Eriksen, L., N. Eriksen and S. Haavaldsen. *The effect of cobalt
ions on the biosynthesis of hemoglobin by rabbit reticulocytes in vitro.* Acta
physiol. scand. 1961. *53.* 300—307. — The effect of cobalt ions on
the biosynthesis of hemoglobin by rabbit reticulocytes in vitro has
been studied. It is shown that cobalt ions in concentrations of ap-
proximately 10^{-3} M almost completely inhibit the formation of
heme, while the formation of globin is unaffected or slightly stimu-
lated. The inhibiting effect on heme formation is found to be due to
inhibition of the formation of the tetrapyrrolic intermediates in heme
formation, while the conversion of the 8 carboxylic intermediate by
stepwise decarboxylation and finally dehydrogenation and iron in-
corporation is unaffected. The implications of the above findings for
the understanding of the effect of cobalt ions on the erythropoiesis are
discussed, and it is suggested that the polycythemia induced by cobalt
ions may be due to a toxic effect of the metal on hemoglobin
formation with a secondary stimulation of red cell formation. The
possibility that cobalt may exert a directly stimulating effect on
the formation of erythropoietine is not, however, excluded.

It has long been known that cobalt salts when administered in small doses to
man or experimental animals, cause development of a polycythemia. How this
effect is exerted is not known, although a series of suggestions have been put
forth (for details see Grant and Root 1952).

The finding of Mcrell, Savoie and London (1958), that cobalt ions in low
concentrations do inhibit the incorporation of ^{14}C-labelled glycine into the
heme group of hemoglobin in red bone marrow cells in vitro, while the incorpo-
ration of glycine into globin was unaffected or slightly stimulated, is of great

interest and might give some clue to the understanding of the effect of cobalt on the erythropoiesis. If as assumed by MORELL *et al.* the decreased incorporation of added glycine into heme actually represents a decreased formation of the heme group, the finding of GOLDWASSER *et al.* (1957) that the erythropoietine titer of plasma from cobalt polycythemic rats is increased in relation to normal rat plasma, might be due to the formation of red cells poor in hemoglobin with secondary increase in the erythropoietine titer. Since however, MORELL *et al.* used only ^{14}C-labelled glycine as marker, it could not be definitely stated whether the in vitro effect of cobalt observed represented an inhibition of heme formation or whether it only meant that the utilization of added glycine for heme formation was inhibited, while heme formation as such went on with unaltered speed. We therefore undertook the following experiments to get more definite information on the effect of cobalt on hemoglobin biosynthesis.

Material and methods

Reticulocyte rich blood was obtained from rabbits made anemic by daily injections of 25 mg phenylhydrazine for 5 days and killed on the 7th day.

The incubation technique and the methods used have been described in details elsewhere (ERIKSEN 1955 a, b, 1956).

As tracers were used glycine labelled in the methyl group and acetate labelled in the carboxyl group with ^{14}C, and ferric chloride labelled with ^{55}Fe. The specific activities of the tracers were glycine-2-^{14}C 2 mc/mmole, acetate-1-^{14}C 1 mc/mmole and FeCl$_3$ 50 mc ^{55}Fe/g Fe. All the isotopes have been obtained from Amersham, England.

Experimental and results

1) The effect of Co^{++} on the incorporation of glycine-2-^{14}C into heme and globin

Two series of bottles were incubated in air for 1 hour at 37° C. Each bottle contained 5 ml cells suspended in 2 volumes of 0.9 % NaCl + 2 μc ^{14}C-glycine + FeCl^3 (90 μg Fe). To series 2 cobalt was added as cobaltous chloride to a final concentration of 3×10^{-3} M.

At the end of the incubation the cells were washed twice with 2 volumes of 0.9 % NaCl and lyzed by freezing and thawing, and finally diluted with 2 volumes of distilled water. The lysates were spun in the Spinco for 90 min at 40,000 r. p. m. and the supernatant used for hemin and globin preparation.

Hemin and globin were plated and counted in the usual way (ERIKSEN 1956) and the activity calculated in infinitely thick layers.

As seen from Table I, Co^{++} almost completely inhibits the incorporation of glycine into heme, while the incorporation into globin is slightly stimulated in complete accordance with the results of NORELL *et al.* (1958).

2) The effect of Co^{++} on the incorporation of ^{55}Fe and ^{14}C-acetate into heme

Experiments similar to those described above were performed. However, as tracers were used carboxyl labelled acetate and ^{55}Fe labelled ferric chloride.

Table I. *The effect of cobalt on the incorporation of* ^{14}C-*labelled glycine into heme and globin by rabbit reticulocytes in vitro*

Time in min	Medium	c/min infinitety thick	
		Hemin	Globin
60	Co^{++}	1	18.6
	control	386.3	13.2

Table II. *The incorporation of* ^{55}Fe *and* ^{14}C-*labelled acetate into heme by rabbit reticulocytes in vitro with and without cobalt added*

Time in min	Medium	c/min/mg	
		^{14}C-Hemin	Hemin-^{55}Fe
60	Co^{++}	20.7	147.1
	control	333.7	2,247.1

Table III. *The effect of cobalt on the formation of free protoporphyrin by rabbit reticulocytes in vitro*

Time in min	μg protoporphyrin	
	Co^{++}	control
0	9.1	9.1
60	6.5	45.2

As seen from Table II, Co^{++} almost completely inhibits the incorporation of both iron and acetate into heme, thus there is very good evidence for the assumption that the decreased incorporation of glycine into heme described by MORELL *et al.* and in the above experiment is due to a true inhibition of heme formation.

3) *The effect of* Co^{++} *on the formation of free protoporphyrin*

Cells suspended in 0.9 % NaCl/glycine (0.03 M) were incubated in air, and the amount of free protoporphyrin was measured at zero time and after 1 hour of incubation with and without cobalt added. As seen from Table III, the amount of free protoporphyrin is slightly lower after 1 hour of incubation than at zero time in the cobalt series, while in the control the amount of protoporphyrin has increased to a value of approximately 5 times the zero value. Thus the formation of protoporphyrin is strongly inhibited by cobalt ions.

Table IV. The effect of cobalt on heme formation by rabbit reticulocytes in vitro when ^{14}C-labelled protoporphyrin has been accumulated in the cells prior to the addition of cobalt

Time min	Series	μg proto	c/min/μg proto	c/min/mg	
				^{14}C-Hemin	Hemin-Fe
0	Cells preinc. in 0.9 % NaCl/glycine (0.03 M) + 1-^{14}C-acetate	11.2	187.1	180.5	—
30	Controls I:FeCl$_3$ III:^{55}FeCl$_3$	15.9	134.2	275.7	4,500.1
	Co^{++} 3 × 10^{-3}M II:FeCl$_3$ IV:^{55}FeCl$_3$	9.9	173.4	179.4	150.0

4) The effect of Co^{++} on heme formation when free protoporphyrin or other possible tetra-pyrrolic intermediates in heme formation have been accumulated in the cells prior to the addition of cobaltous chloride

The slight drop in the amount of free protoporphyrin in the cobalt series in the previous experiment, might indicate that some of the preformed proto-porphyrin had been utilized for heme formation. To test this possibility the following experiment was performed.

Cells suspended in 0.9 % NaCl/glycine (0.03 M) with ^{14}C-labelled acetate as tracer were incubated for 30 min in air. Under these conditions the only possible tetrapyrrolic intermediate in heme formation labelled with ^{14}C and present in measurable amounts in the cells after the incubation would be proto-porphyrin (ERIKSEN 1955 a, 1956, ERIKSEN, ERIKSEN and HAAVALDSEN 1960).

At the end of the incubation the cells were centrifuged down, washed twice with 2 volumes of 0.9 % NaCl and resuspended in 0.9 % NaCl/glycine, and 4 series were incubated further in air for 30 min. To series I and II inactive ferric chloride was added and to series III and IV ferric chloride labelled with ^{55}Fe. The final concentration of iron in the incubation bottles was 600 μg % Fe. To series II and IV cobaltous chloride was added to a final concentration of 3×10^{-3} M.

The amount of free protoporphyrin and the specific activities of free proto-porphyrin and hemin were measured before and after the second incubation.

As seen from Table IV, no or negligible amounts of heme have been formed in the cobalt series, while in the control a substantial amount of heme has been formed as judged both from the amount of ^{55}Fe incorporated and the increase in the specific activity of ^{14}C labelled hemin.

Table V. The effect of cobalt on heme formation by rabbit reticulocytes in vitro when ^{14}C-labelled intermediates corresponding to uro- and coproporphyrin have been accumulated in the cells prior to addition of cobalt

Time in min	Series	Atmosphere	Type of porphyrins found			c/min/mg Hemin
			uro	copro	proto	
0	Cells preinc. in plasma/glycine (0.03 M) + 1-^{14}C-acetate.	N$_2$/CO$_2$	2 +	4 +	2 +	104.5
30	Control	air	—	trace	6 +	133.2
	Co^{++} 3 × 10^{-3}M	air	—	trace	6 +	139.5

As shown in the preceding paper (ERIKSEN 1961), preformed protoporphyrin is only poorly utilized for heme formation in cell systems of the type used in these experiments. Thus, even though cobalt may inhibit the formation of heme from iron and the true intermediate corresponding to protoporphyrin, it must, especially since the drop in the specific activity of free protoporphyrin in the cobalt series is lower than in the control, have an inhibitory effect at some earlier step in the sequence of events leading to heme. Information on the possible step in heme formation inhibited by cobalt ions was sought in the following way.

Cells suspended in 2 volumes of plasma/glycine (0.03 M) + carboxyl labelled acetate were incubated for 60 min in a stream of N$_2$/CO$_2$ containing approximately 3 % oxygen. Under these conditions intermediate tetrapyrroles having from 8—2 carboxyl groups will accumulate in the cells (ERIKSEN 1955 a, 1956, ERIKSEN et al. 1960). At the end of the incubation cells and plasma were separated by centrifugation and the cells washed twice with 0.9 % NaCl. The main part of the cells were suspended in 2 volumes of 0.9 % NaCl/glycine (0.03 M) containing iron as ferric chloride in a concentration of 600 μg Fe per cent. The suspension was divided into 2 series and further incubated in air for 30 min. To one of the series cobalt was added as cobaltous chloride to a final concentration of 3 × 10^{-3} M.

The specific activity of hemin was measured before and after the second incubation. The total amount of the free tetrapyrrolic intermediates was not measured. However, all the intermediate tetrapyrroles were extracted with ethylacetate/glacial acetic acid (4/1) and converted into porphyrin as described elsewhere (ERIKSEN 1956). The porphyrins were adsorbed to small talc columns, washed and eluted with 10 % NH$_3$/acetone (3/7), applied to paper and chromatographed according to ERIKSEN (1953).

As seen from Table V, tetrapyrroles giving rise to porphyrins having from 8—2 carboxylic groups were present in the cells prior to the second incubation, and the main porphyrin behaved as coproporphyrin. After the second incuba-

Table VI. *The effect of cobalt on the incorporation of ^{55}Fe into heme by rabbit reticulocytes in vitro when intermediates corresponding to uro- and coproporphyrin have been accumulated in the cells prior to the addition of cobalt*

Time in min	Series	Atmos- phere	Type of porphyrins found			c/min/mg Fe
			uro	copro	proto	
0	Cells preinc. in plasma/gly-cine (0.03 M)	N_2/CO_2	2 +	4 +	2 +	
30	Control	air	—	trace	6 +	3,332.1
	$Co^{++}\ 3 \times 10^{-3}M$	air	—	trace	6 +	3,528.0

tion the porphyrin pattern has changed completely and in the same way in both series. The main porphyrin is now behaving as protoporphyrin, copro-porphyrin is present in negligible amounts only and no trace of porphyrins having more than four carboxyl groups are found. It is further seen from the table that the specific activities of the hemins have increased approximately 30 % in both series.

Since neither the porphyrin pattern nor the amount of the newly formed heme are different in the cobalt series from that of the control, it becomes evident that cobalt has no effect neither on the conversion of the tetrapyrrolic intermediates into the true heme precursor nor on the insertion of iron into this compound.

To exclude the possibility that the ^{14}C activity found in hemin should be acci-dental and due to some unspecific contamination of the hemin isolated, the experiment was repeated, and as a control was used 2 series in which ^{55}Fe was used as tracer. The results were completely identical with those cited, and as shown in Table VI, the amount of heme formed during the second incubation as judged from the amount of ^{55}Fe incorporated is the same whether cobalt has been added or not.

We have previously shown that the heme formed is true protopheme (ERIKSEN 1955 a, 1956), thus there is no reason to believe that the ^{14}C and ^{55}Fe activities of the hemins should be due to admixture of trace amounts of uroheme or coproheme, an assumption which in itself does not seem very probable on the basis of the above experiments.

Discussion

Our findings are in complete accordance with those of MORELL *et al.* (1958) and show that the inhibition of ^{14}C-glycine incorporation into the heme group by cobalt ions described by these authors is due to the inhibition of heme formation. Our data also show that the inhibitory effect of cobalt on heme for-mation is due to a block in the formation of the tetrapyrrolic intermediates in heme formation at some step prior to uroporphyrinogen, while the conversion

of this latter into the copro- and proto-type intermediates and the final insertion of iron into the latter is unaffected.

The results cited in Table IV and V also support our finding in the preceding paper (ERIKSEN 1961) that preformed protoporphyrin is not or only poorly utilized for heme formation in hemoglobin forming systems of the type described. The above results also strongly support our previous finding that protoporphyrin and globin are formed by completely independent processes and can be taken as support for the assumption that globin acts as an acceptor for the normal protoporphyrin type intermediate in heme formation or for heme itself (ERIKSEN 1955 a, 1956, 1957, 1960). The latter possibility has been discussed in details elsewhere (ERIKSEN 1960), and the reader is referred to this paper.

It is obviously too early to extend the above in vitro findings to the situation in whole animals. It is, however, interesting that Davis and FIELDS (1958) found that the red cells produced in cobalt induced polycythemia in man were hypochromic, indicating that the formation of new red cells was less affected than the formation of the hemoglobin molecule.

SAIKKONEN (1959) found that cobalt administration to rat caused a significant increase in coproporphyrin excretion parallel with a small but significant drop in the hemoglobin concentration in peripheral blood. After 10—14 days a true polycythemia developed. A series of other poisonous effects were also described, and SAIKKONEN pointed upon the similarity of the action of cobalt to heavy metal poisoning.

The findings of DAVIS and FIELDS (1958) and SAIKKONEN (1959) when taken together with our own in vitro findings, could be taken as support for the assumption that the increased erythropoietine titer in cobalt polycythemic rats described by GOLDWASSER et al. (1957) is secondary to the formation of red cells poor in hemoglobin. They do not, however, exclude the possibility that cobalt apart from the toxic effect described above, do exert a directly stimulating effect on the erythropoietine forming tissues, and further experiments are needed.

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The Formation of Coproporphyrin III and I by Rabbit Reticulocytes Under Hypoxic Conditions in Vitro

By

LEIF ERIKSEN, NORA ERIKSEN and SYNNÖVE HAAVALDSEN

Received 11 June 1961

Abstract

ERIKSEN, L., N. ERIKSEN and S. HAAVALDSEN. *The formation of coproporphyrin III and I by rabbit reticulocytes under hypoxic conditions in vitro.* Acta physiol. scand. 1961. *53.* 308—314. — It has been shown that tetrapyrroles having from 4 to 8 carboxylic groups of both the III and I isomer series are formed by rabbit reticulocytes in vitro under hypoxic conditions. The tetrapyrroles of the III series are rapidly converted into protoporphyrin under aerobic conditions, while the tetrapyrroles of the I series are converted into coproporphyrinogen I. The implications of these findings have been discussed and it is suggested that an 8-carboxylated openchained tetrapyrrolic intermediate of the I series is normally formed by a deaminase, the so-called porphobilinogenase, and that this tetrapyrrole is condensed spontaneously into uroporphyrinogen I if this condensation is not hindered by a second enzyme, the so-called isomerase. The possible effect of the latter is discussed and a scheme for the formation of ringformed tetrapyrroles of both the III and I series has been suggested. It is further suggested that the formation of ringformed tetrapyrroles of the I series is due to inhibition or blockage of the isomerase with spontaneous condensation into uroporphyrinogen I of the openchained tetrapyrrole of the I series formed by the deaminase, uroporphyrinogen I being rapidly converted by the decarboxylases present into coproporphyrinogen I. The accumulation of the latter when the atmosphere is changed into air, shows that the enzymes responsible for the conversion of coproporphyrinogen into protoporphyrin are highly specific and can utilize only coproporphyrinogen III and explains why porphyrins having less than 4 carboxylic groups have been found in nature only as isomers of the III series.

In a series of previous papers we have shown that tetrapyrrolic intermediates in heme formation tend to accumulate in rabbit reticulocytes under hypoxic conditions in vitro (ERIKSEN 1955, 1956, ERIKSEN, ERIKSEN and HAAVALDSEN 1960).

Since under physiological conditions both man and rabbit excrete small amounts of at least three different coproporphyrin precursors (ERIKSEN, ERIKSEN and HAAVALDSEN 1961 a) giving rise to the coproporphyrin isomers III (IV), I and trace amounts of a porphyrin behaving as the II isomer of coproporphyrin (ERIKSEN 1958 a, ERIKSEN et al. 1961 a, b), the possibility did exist that these coproporphyrin precursors were formed during the biosynthesis of heme. To test this possibility a study of the isomer pattern of coproporphyrin obtained from rabbit reticulocytes incubated in plasma/glycine under highly hypoxic conditions was undertaken.

Experimental and Results

The technique used for the preparation of blood rich in reticulocytes from rabbits has been described in detail elsewhere (ERIKSEN 1956).

The cells were suspended in 2 volumes of plasma/glycine (0.032 M) and incubated in a stream of N_2/CO_2 containing less than 3 per cent oxygen.

The accumulated tetrapyrroles were extracted with ethylacetate/glacial acetic acid (4:1), converted into porphyrins by extraction with 3 N HCl acid (ERIKSEN 1956), and separated by means of paper chromatography (ERIKSEN 1953). The zone corresponding to coproporphyrin was cut out, eluted with 3 N HCl acid, adsorbed to small talc columns, eluted with 10 per cent NH_3/acetone (3/7), and finally the purified coproporphyrin fraction was applied to paper and chromatographed in our coproporphyrin isomer separating system (ERIKSEN, 1958 b).

As seen from Fig. 1, only the III isomer of coproporphyrin is present in measurable amounts. A slight tailing might indicate the presence of trace amounts of the I isomer. However, the isomer pattern is completely different from that found in the urine of normal man and rabbit in which, although the III isomer is quantitatively dominating, a considerable amount of the I isomer is also present together with trace amounts of a possible II isomer (ERIKSEN et al. 1961 a, b).

As mentioned above, the slight tailing observed might be due to the presence of trace amounts of the I isomer. To test this possibility the following experiments were undertaken.

Fig. 1. Isomer chromatogram of the coproporphyrin fraction obtained from reticulocytes incubated in plasma/glycine under hypoxic conditions. S = sample, III = coproporphyrin III, I = coproporphyrin I.
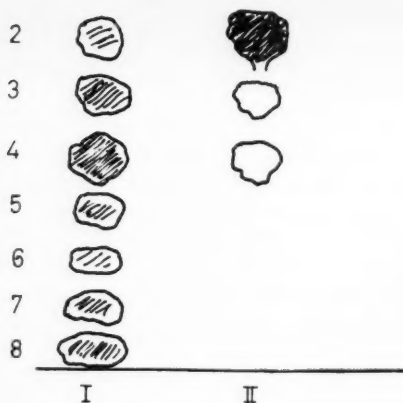
Fig. 2. Chromatogram of porphyrins found in rabbit reticulocytes after preincubation in plasma/glycine under hypoxic conditions (I), and reincubation in NaCl/glycine in air (II). System lutidine/H$_4$O 5/3 + NH$_3$, unsaturated.

Rabbit reticulocytes incubated as described above were suspended in 2 volumes of saline/glycine (0.032 M) and incubated in air for 30 min, and the whole incubate was worked up on porphyrins in the usual way.

Under these conditions all the tetrapyrroles of the III series would be converted into protoporphyrin IX (ERIKSEN 1955, 1956, ERIKSEN et al. 1960), while the I isomers would stop at the coproporphyrinogen level (ERIKSEN, HAAVALDSEN and ERIKSEN 1960). Thus, if tetrapyrroles having 4 or more carboxylic groups were present prior to the aerobic incubation, all the I isomers should be trapped as coproporphyrinogen I after the aerobic incubation.

As seen from Fig. 2, approximately all the porphyrin present after aerobic incubation is present as a dicarboxylic porphyrin which has been identified as protoporphyrin.

Small amounts of porphyrins behaving as 3 and 4 carboxylic are also present. The amount of these latter porphyrins was very small and was difficult to handle even with the micro technique described elsewhere (ERIKSEN et al. 1961 b). To get information about the isomeric nature of the coproporphyrin without losing appreciable amounts of porphyrin, a chromatogram was turned 90° and chromatographed in our coproporphyrin isomer separating system (ERIKSEN 1958 b). As seen from Fig. 3, the coproporphyrin spot has split into two distinct spots corresponding to the III and I isomers. The amount of porphyrin as judged from the colour and fluorescence of the spots is approximately equal. Thus the ratio of I/III of coproporphyrin has changed from approximately zero to 1. The presence of some III isomer after reincubation in air most probably is due to oxidation of the true intermediate during the aerobic incubation.

To exclude the possibility that the I isomer had been formed during the aerobic incubation, the experiment was repeated. However, this time cobaltous
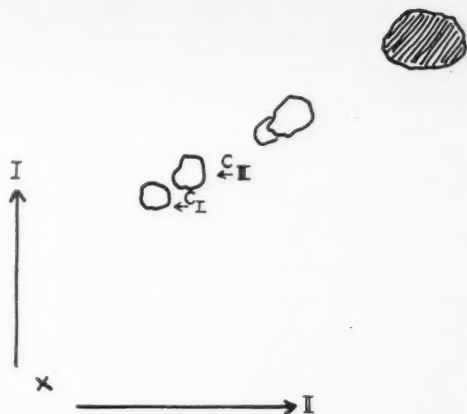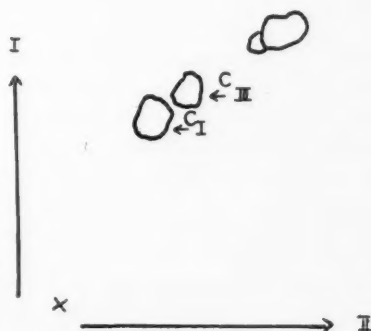
Fig. 3. Twoways chromatogram of porphyrins found in reticulocytes preincubated in plasma/glycine under hypoxic conditions and reincubated in NaCl/glycine in air. System I, lutidine/H_2O 5/3 + NH_3, unsaturated, system II, lutidine/H_2O 5/2 + NH_3, saturated.

Fig. 4. Twoways chromatogram of porphyrins found in reticulocytes preincubated in plasma/glycine under hypoxic conditions and reincubated in NaCl/glycine in air with Co^{++} added. System I, lutidine/H_2O 5/3 + NH_3, unsaturated, system II, lutidine/H_2O + NH_3, saturated.

chloride was added to the aerobic incubation bottles to a final concentration of 3×10^{-3} M. Cobalt ions of this concentration inhibit almost completely the formation of the tetrapyrrolic intermediates in heme formation, while it has no effect on the conversion of these intermediates into protoporphyrin and heme (ERIKSEN 1960, ERIKSEN et al. 1961 c). Thus, if tetrapyrroles of the I series are present after the aerobic incubation, these must origin from tetrapyrroles formed during the hypoxic incubation and having 4 or more carboxylic groups.

As seen from Fig. 4, the isomer pattern of coproporphyrin is identical with that obtained in the above experiment. Thus there is very good reason to assume that tetrapyrroles of both the III and I series have been formed under hypoxic conditions. The amount of the tetrapyrroles of the I series is extremely small as compared to that of the III series.

Discussion

As shown by BOGORAD (1958), two different enzymes seem to be needed for the conversion of porphobilinogen into uroporphyrinogen III: a deaminase (porphobilinogenase) giving rise to a polypyrrole with a sidechain sequence corresponding to the I series, and an "isomerase" directing the final condensation in such a way that one (or three) of the pyrroles is rotated to give a sidechain sequence of the III series prior to the condensation into uroporphyrinogen.

Uroporphyrinogen I can not be utilized as a substrate for the isomerase (MAUZERALL and GRANICK 1958), thus it is reasonable to assume that the substrate is an openchained tetrapyrrole with a sidechain sequence corresponding to the I series, and which, if the isomerase is destroyed by heat (GRANICK and MAUZERALL 1958, BOOIJ and RIMINGTON 1957) or limited by accumulation of product as in the above experiments, would condense chemically into uroporphyrinogen I.

Since the uroporphyrinogen decarboxylase responsible for the conversion of uroporphyrinogen into coproporphyrinogen does not distinguish between the different isomers (MAUZERALL and GRANICK 1958), any uroporphyrinogen I formed would be expected to be converted into coproporphyrinogen I when the block in protoporphyrin formation due to hypoxia is released during the aerobic incubation, similar to what has been found above.

Our findings may be explained according to the following scheme for protoporphyrin formation from porphobilinogen (Fig. 5).

According to this scheme the isomerase acts by blocking the free α-carbon in ring A and loosening the linkage between the other α-carbon and the methylene bridge carbon, allowing the A ring to rotate and the methylene carbon in the D ring to link to the carbon atom set free by the isomerase, thus giving rise to a tetrapyrrole of the III series which finally can condense into uroporphyrinogen III. If the isomerase is destroyed or limited, the openchained tetrapyrrole would be expected to condense almost spontaneously to uroporphyrinogen I.

The above scheme is supported by the finding of BOGORAD (1958) that the deaminase/isomerase system under certain conditions can be split into two subfractions, fraction I being capable to form uroporphyrinogen I with porphobilinogen as a substrate, while fraction II can not use porphobilinogen as a substrate at all. However, addition of fraction II to fraction I gives rise to the formation of uroporphyrinogen III when incubated with porphobilinogen. The deaminase thus seems to be the enzyme responsible for the condensation of the porphobilinogen molecules into the tetrapyrrole, which, if not directed by the isomerase, will condense into uroporphyrinogen I.

Since the isomerization and condensation takes place without the release of formaldehyd or any other one carbon compound (LOCKWOOD and BENSON 1960), it means that the isomerization, that is the migration of one bridge carbon atom from one α-position to another via the N-atom, most probably takes place after the formation of the immediate tetrapyrrolic precursor to uroporphyrino-

Fig. 5

$$\text{Porphobilinogen} \xrightarrow[-4\,NH_3]{\text{Deaminase}}$$

Porphobilinogen

Nonenzymic

↓ Isomerase

Uroporphyrinogen I

↓ Dec.

Coproporphyrinogen I

Uroporphyrinogen III

↓ Dec.

$$\text{Coproporphyrinogen III} \xrightarrow[\text{Dehydr.}]{\text{Dec.}} \text{Protoporphyrin IX} \longrightarrow \text{Heme}$$
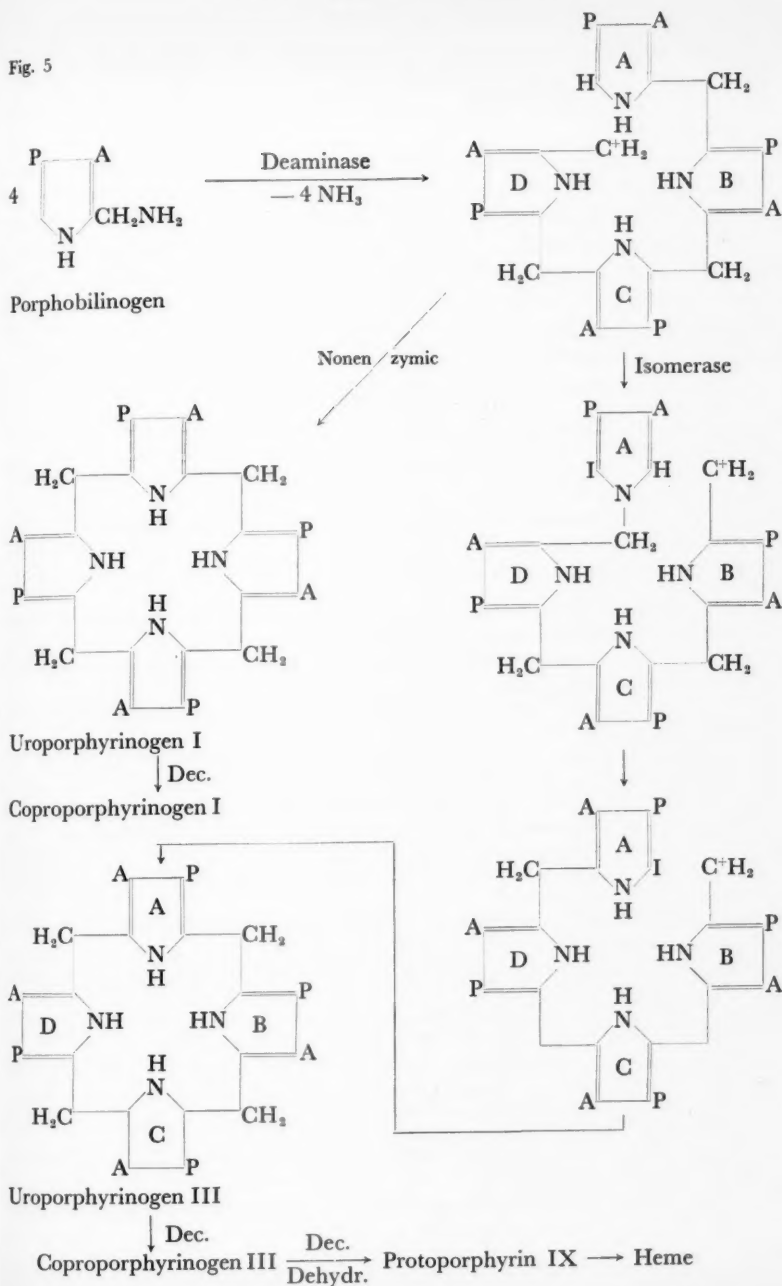
gen, and which, if the isomerase is not present, will condense spontaneously into uroporphyrinogen I. The possibility that the condensation takes place prior to the isomerization seems to be definitely excluded by the finding of GRANICK and MAUZERALL (1958) that uroporphyrinogen I can not serve as a substrate for the isomerase.

The mechanism suggested by BULLOCK *et al.* (1958) for the chemical formation of coproporphyrin III, assuming a migration of the methylene group from the D ring to the free *a*-carbon of the A ring, thus changing the sidechain sequence with final addition of the B and C rings to give the III isomer, can not explain the formation of the I isomer when the isomerase is destroyed or inhibited, since the tetrapyrrole formed would have a sidechain sequence identical to that of the III series, and one (or three) of the pyrrole rings had to be rotated prior to condensation to give uroporphyrinogen I, a possibility which does not seem very likely.

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Evidence for Energy-Requiring Processes in Histamine Release and Mast Cell Degranulation in Rat Tissues Induced by Compound 48/80

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Abstract

DIAMANT, B. and B. UVNÄS. *Evidence for energy-requiring processes in histamine release and mast cell degranulation in rat tissues induced by Compound 48/80*. Acta physiol. scand. 1961. *53*. 315—329. — Histamine release from rat lung tissue and degranulation of rat mesentery mast cells were produced *in vitro* by Compound 48/80. Both processes were shown to be blocked by anoxia and by inhibitors of oxidative phosphorylation. Preincubation with glucose counteracted this inhibition. The results indicate that histamine release and mast cell degranulation require energy which can derive from glucose metabolism.

Most authors agree that anaphylactic histamine release from sensitized guinea-pig lung tissue *in vitro* requires oxygen, since anoxic conditions and metabolic inhibitors, such as dinitrophenol, azide and cyanide (according to some reports, this occurs only in rather high concentrations) depress such release (PARROT 1942, MONGAR and SCHILD 1957, MOUSSATCHÉ and PROVOUST-DANON 1958, CHAKRAVARTY 1960). It was stated, however, that antigen-induced histamine release from sensitized rat lung tissue *in vitro* was not influenced by anoxia (CHAKRAVARTY 1959). On the other hand, degranulation of mast cells from sensitized rats was blocked by metabolic inhibitors, including those which are assumed to uncouple oxidative phosphorylation and to block respiratory enzymes (HÖGBERG and UVNÄS 1960, MOTA and ISHII 1960).

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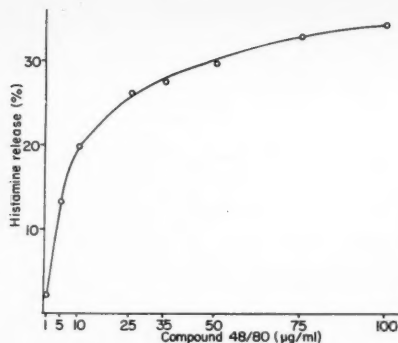
Fig. 1. Dose-response curve for Compound 48/80-induced histamine release from rat lung tissue under oxygen in presence of glucose (5.6 mM). Histamine release computed in per cent of total histamine content. Spontaneous release deducted from all values.

As opposed to these results for sensitized rat tissues, degranulation of non-sensitized rat mast cells, caused by extracts from Ascaris suis and Cyanea capillata, is blocked by metabolic inhibitors (UVNÄS et al. 1960, UVNÄS 1960); and the histamine release from non-sensitized rat lung tissue in vitro, due to Ascaris extracts, is blocked by anoxic conditions (DIAMANT 1960, 1961).

Views diverge very considerably regarding the operative mechanism of the polymer amine, Compound 48/80. Its histamine releasing action is regarded as being independent of oxygen (rat lung, CHAKRAVARTY 1959; cat paw, CHAKRA-VARTY, HÖGBERG and UVNÄS 1959). In fact, histamine release from guinea-pig lung tissue, due to Compound 48/80, was stated to be enhanced by oxygen lack and by metabolic inhibitors (MONGAR and SCHILD 1957). Recently, however, it was reported that metabolic inhibitors and anoxia reduce the histamine release from cat skin (WESTERHOLM 1960, 1961) and rat tissues (MOTA and ISHII 1960).

Degranulation of rat mast cells induced by Compound 48/80 was reported to be blocked by metabolic inhibitors (JUNQUEIRA and BEIGUELMAN 1955, HÖGBERG and UVNÄS 1957, 1960, MOTA and ISHII 1960). On the other hand, the incubation of rat mast cells under nitrogen did not abolish degranulation (HÖGBERG and UVNÄS 1960).

Since it was shown (DIAMANT 1960) that the presence of glucose enhanced the histamine release, induced by extracts of Ascaris suis, from rat lung tissue, and since similar observations were reported regarding the histamine release induced by Compound 48/80 from cat skin in vitro (WESTERHOLM 1960), it was suggested that the divergent results of earlier investigations on the effect of Compound 48/80 could, in part, be attributed to the presence of glucose. Consequently, in this report, we have re-investigated, with regard to the effect of glucose, the influence of metabolic inhibitors and anoxia on the histamine release and the mast cell degranulation induced by Compound 48/80 in the rat.
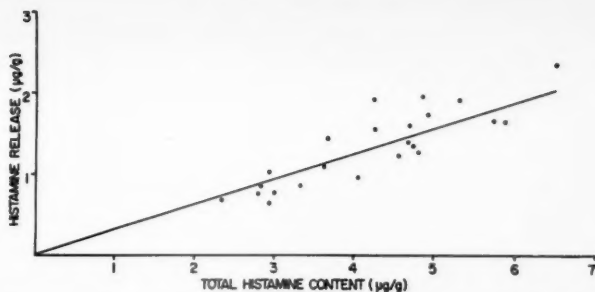
Fig. 2. Histamine release from rat lung tissue under nitrogen in presence of glucose (5.6 mM) induced by Compound 48/80 (35 μg/ml) as related to the total histamine content in 23 different experiments. All values given as μg histamine base per g of wet lung tissue. Spontaneous release deducted from all values.

In previous communications from this laboratory (Högberg and Uvnäs 1960), evidence has been presented to support the view that the mode of action of Compound 48/80 is, in principle, the same as that of antigen and histamine releasers extracted from natural sources, such as Ascaris and Cyanea. The present investigation will provide further evidence to strengthen this view.

Methods and Materials

Incubation medium

In all experiments, we used, as incubation medium, a solution containing NaCl (154 mM), KCl (2.7 mM) and CaCl$_2$ (anhydrous, 0.9 mM) buffered with Sörensen phosphate buffer (67 mM), 10 per cent v/v. The pH of the medium was between 7.0 and 7.2 in the experiments with isolated rat lung tissue and 6.9—7.4 in those on the degranulation of rat mesentery.

Histamine release from isolated rat lung tissue

The technique used was similar to that described by Diamant (1961), but with slight modifications. The lungs were excised from male and female rats, weighing 250—400 g. In each experiment 3—6 animals were used. After removal of visible bronchi, all lung lobes were pooled. They were then cut into smaller pieces (1—2 mg). After careful washing with the incubation medium, the lung tissue was blotted dry. It was subsequently divided into smaller parts by weighing. The weight of each sample was usually between 500—600 mg (in no case less then 400 mg). Thus, a solution containing no glucose was used for the entire preparation of the lung samples. This could be accomplished without affecting the sensitivity of the tissue to Compound 48/80, since preliminary results had shown that there was no difference in the histamine release in a medium containing glucose, irrespective of whether or not the lung tissue had been treated, before incubation, with a solution containing glucose (5.6 mM).

The lung samples were incubated and shaken in stoppered Erlenmeyer flasks in a Warburg apparatus at 37° C. Unless otherwise indicated, glucose or other carbohydrates, when tested, were present in the Erlenmeyer flasks from the beginning of the incubation.
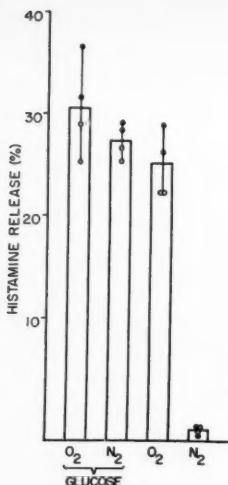
Fig. 3. The effect of glucose (5.6 mM) on histamine release induced by Compound 48/80 (35 µg/ml) from rat lung tissue under the influence of oxygen and nitrogen. Filled and open circles represent the individual values of double tests from two different experiments. All release values computed in per cent of total histamine content. Spontaneous release deducted from all values.

Oxygen or nitrogen gas was bubbled through, via syringe needles, during the first 15 min of incubation. Compound 48/80 was then added (with a final concentration of 35 µg/ml, except where otherwise stated), and, at the same time, the syringe needles were withdrawn above the surface of the solutions. Then, 20 min after the addition of Compound 48/80 the incubation fluids were withdrawn. In the text, this incubation procedure is referred to in an abbreviated form, i. e. 15 min glucose N_2 + 20 min 48/80 N_2. Similar abbreviations are also used for other incubation procedures of lung tissue as well as of isolated mesentery. The remaining histamine in each lung tissue sample was extracted by heating to 100° C on a water bath for 10 min. Histamine was assayed on atropinized guinea pig ileum. The contractions were blocked by antihistamines, showing that the contractions were due to histamine. All values given are corrected for the spontaneous histamine release.

When dinitrophenol (DNP) was studied, the lung samples were preincubated with DNP for 15 min. During this time no gases were bubbled through the incubation fluid. Introduction of oxygen or nitrogen and eventually addition of glucose then followed. Fifteen min later Compound 48/80 was added, and after a further 20 min, the incubation fluids were withdrawn.

All concentrations of substances mentioned in the text refer to the final concentration in the incubation medium.

Degranulation of mast cells in isolated rat mesentery

The technique used was the same as that described by Högberg and Uvnäs (1957, 1960). The mesentery pieces were incubated in open bowls on a water bath at 37° C for 20 min after the addition of Compound 48/80 (0.5 µg/ml, unless otherwise indicated). Where inhibitors and glucose were investigated, the mesentery was preincubated together with these substances before the addition of Compound 48/80, as described in the text.

Table I. *The effect of oxygen, nitrogen and glucose (5.6 mM) on Compound 48/80 (35 µg/ml) induced histamine release from rat lung tissue in vitro. Histamine release computed in per cent of total histamine content. Spontaneous release deducted from all values*

Exp. no.	Histamine release			
	(A)	(B)	(C)	(D)
1	34.1	27.7	28.8	1.0
2	27.1	22.3	26.1	1.4
3	30.7	28.6		
4	36.8		35.3	1.9
5	26.7	19.4	25.9	1.4
6	32.3	29.3	30.4	2.3
Mean and standard error of mean:....	31.3 ± 1.6	25.5 ± 2.0	29.3 ± 1.7	1.6 ± 0.23
Significance of difference of mean from O_2+glucose (*t*-test)		$P = 0.05-0.01$	$P = > 0.05$	$P = < 0.001$

Incubation procedures: A: 15 min glucose O_2 + 20 min 48/80 O_2
                        B: 15 min O_2 + 20 min 48/80 O_2
                        C: 15 min glucose N_2 + 20 min 48/80 N_2
                        D: 15 min N_2 + 20 min 48/80 N_2

When the influence of nitrogen or oxygen on the degranulation was investigated, the mesentery pieces were incubated in stoppered Erlenmeyer flasks in a Warburg apparatus at 37° C without shaking. The incubation fluids were nitrogenated or oxygenated for at least 10 min, before the addition of the mesentery pieces. After these had been added, the needles used for gassing were withdrawn so that they were just above the surface of the incubation fluids, in order to avoid mechanical damage of the mesentery cells during incubation. In all the experiments, the mesentery pieces were exposed to Compound 48/80 for 20 min. All values given are the means of duplicates. Further details are given in the text.

Materials

Compound 48/80 was prepared by AB Leo, Hälsingborg, Sweden, and supplied by the courtesy of Dr B. Högberg.

The following carbohydrates were used:

D-glucose, anhydrous (analytical reagent grade, from the Mallinckrodt Chemical Works).
D-galactose (not graded, from Hoffmann-La Roche).
D-fructose + H_2O (not graded, from Hoffmann-La Roche).
Maltose + H_2O (not graded, from Hoffmann-La Roche).
Lactose + H_2O (not graded, from Hopkins and Williams Ltd).
Sucrose (analytical reagent grade, from the Mallinckrodt Chemical Works).

All inhibitors were obtained from standard commercial sources, except for allicin, which was prepared from garlic according to Cavallito and Bailey (1944).
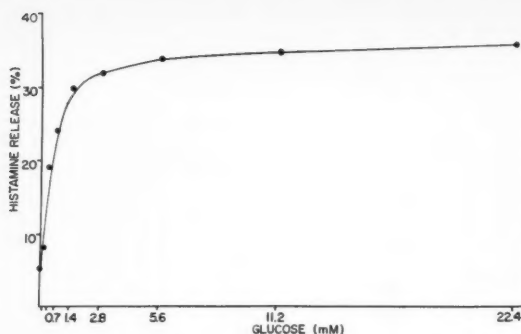
Fig. 4. The effect of glucose (concentrations geometrically decreasing from 22.4 to 0.175 mM) on histamine release induced by Compound 48/80 (35 µg/ml) from rat lung tissue under the influence of nitrogen. Histamine release computed in per cent of total histamine content. Spontaneous release deducted from all values.

Results

A. LUNG TISSUE

Dose response relationship

Guided by the dose response curve in Fig. 1, the histamine dose, chosen for the experiments described under A, was 35 µg/ml. Since the histamine content of the lung tissue varied considerably from animal to animal it was not surprising to find wide variations between the animals in the amount of histamine released by one and the same dose of Compound 48/80 (Fig. 2). On the other hand, the percentual release was fairly constant amounting to 31.4 ± 4.7 per cent (mean and standard deviation in Fig. 2). Consequently, in the following, histamine release is given in per cent of the total histamine content.

Influence of oxygen nitrogen and glucose

Nitrogen was found to strongly depress the histamine release from rat lung tissue, provided that the incubation fluid was glucose-free. In the two experiments shown in Fig. 3, the histamine release caused by Compound 48/80 fell from an average of 31 per cent in an oxygenated medium containing glucose 5.6 mM (1 mg/ml) to 1 per cent in a nitrogenated glucose-free medium. On the other hand, in the presence of glucose, oxygen lack did not significantly lower the histamine release. In glucose-free, but oxygenated medium, the histamine release was slightly reduced as compared with that in the presence of glucose and oxygen. The average values from 6 exp. (Table I) show that, under nitrogen and in the absence of glucose (D), the histamine release was depressed to around 1 per cent, as compared with 31 per cent in oxygenated medium containing glucose (A). Under nitrogen, but in the presence of glucose (5.6 mM) (C), the release was not significantly lowered below that of (A); whereas under oxygen, but in absence of glucose (B), the release fell to about

Table II. The effect of glucose (5.6 mM) on the histamine release from rat lung tissue under nitrogen in vitro, induced by Compound 48/80 (35 µg/ml). Histamine release computed in per cent of total histamine content. Spontaneous release deducted from all values

Exp. no.	A	B	C	D
1	39	8	4	
2	45	3	1	
3	35	8	0	
4	36	12	5	
5	31	7	3	
6	36			26
7	35			23
Mean:	37	8	3	27

Incubation procedures:

A: 15 min glucose N_2 + 20 min 48/80 N_2
B: 15 min N_2 + 20 min 48/80 glucose N_2
C: 15 min N_2 + 20 min 48/80 N_2
D: 15 min N_2 + 15 min glucose N_2 + 20 min 48/80 N_2

24 per cent. This fall was significant ($P = 0.05 - 0.01$); although small compared with the decline in (D).

The minimal concentration of glucose required to appreciably counteract the depressing effect of nitrogen atmosphere was found to be around 0.25 mM (0.045 mg/ml). Above 5.6 mM (normal blood level) increase of glucose concentration did not appreciably enhance the histamine release (Fig. 4).

To obtain the optimal preservative effect of glucose on the histamine-releasing action of Compound 48/80 in nitrogenated lung tissue, preincubation of the glucose with the tissue was required. As shown in Table II, when glucose and Compound 48/80 were added simultaneously to the incubation medium (B), the histamine release averaged only 8 per cent as against 3 per cent in the absence of glucose (C); whereas 37 per cent histamine was released when glucose was added 15 min before Compound 48/80 (A). The minimal preincubation time, needed for obtaining the full glucose effect, could not be determined with the technique used; but 15 min preincubation sufficed to produce a maximal effect. The quantitative evaluation of the preservative effect of glucose was complicated by the fact that incubation of the lung tissue in nitrogenated, glucose-free medium, caused a progressive decline in the restorative ability of glucose. During 15 min incubation under nitrogen in the absence of glucose, this decline was, however, too low to affect the validity of our results (see (D) in Table II).
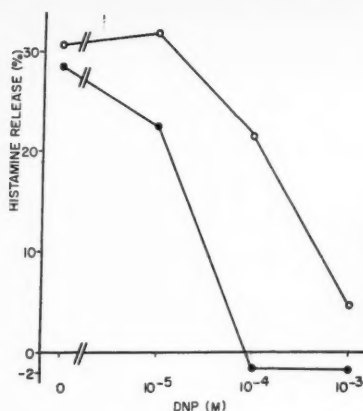
Fig. 5. The effect of DNP on histamine release induced by Compound 48/80 (35 μg/ml) from rat lung tissue under oxygen in presence (○–○–○–○) and absence (•–•–•–•) of glucose (5.6 mM). Histamine release computed in per cent of total histamine content. Spontaneous release deducted from all values.
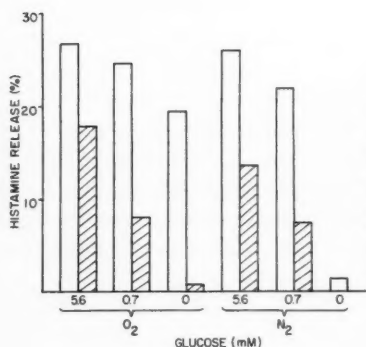

Fig. 6. The effect of DNP (10^{-4}M) on the histamine release induced by Compound 48/80 (35 μg/ml) from rat lung tissue under the influence of oxygen and nitrogen in absence and presence (0.7 and 5.6 mM) of glucose. Unshaded blocks represent histamine release in absence of DNP, and shaded blocks that in presence of DNP. Histamine release computed in per cent of total histamine content. Spontaneous release deducted from all values.

The effect of dinitrophenol

Dinitrophenol (DNP), was observed to block the histamine release in concentrations above 10^{-5}M, provided this reagent was allowed to act on the lung tissue in a glucose-free oxygenated medium. The presence of glucose reduced this inhibitory action of DNP. In the experiment shown in Fig. 5, the histamine release, in the absence of DNP and glucose, was 29 per cent. With DNP 10^{-4}M, no release occurred; with DNP 10^{-4}M + glucose 5.6 mM, 22 per cent histamine was released. Fig. 6 shows that DNP 10^{-4} M reduced histamine release, in the presence of glucose (5.6 and 0.7 mM), to the same extent, both in an oxygenated and in a nitrogenated medium.

Fig. 7. The effect of mono- and disaccharides on the histamine release induced by Compound 48/80 (35 µg/ml) from rat lung tissue under nitrogen. Histamine release computed in per cent of total histamine content. Spontaneous release deducted from all values.
1. Glucose 5.6 mM
2. Galactose 5.6 mM
3. Fructose 5.6 mM
4. Lactose 5.6 mM
5. Sucrose 5.6 mM
6. Maltose 5.6 mM
7. No saccharide present.
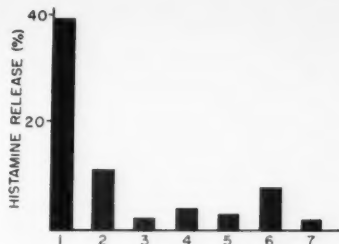
Fig. 8. Degranulating action of Compound 48/80 (•–•–•–• = 2 µg; o–o–o–o = 0.5 µg) in nitrogenated glucose-free medium on mesentery mast cells preincubated under nitrogen for various periods. Abscissa represents preincubation time under nitrogen.
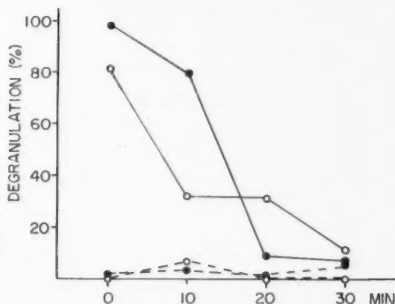
The effect of monosaccharides and disaccharides

The restorative effect on the histamine release under nitrogen seems to be more or less specific for glucose. Out of 6 mono- and disaccharides tested, glucose alone had a marked effect. Galactose and maltose, however, were observed to exert a slight effect, whereas fructose, lactose and sucrose were inactive (Fig. 7).

Unfortunately, pro analysi products of most of the sugars, including galactose and maltose, were not available in our case. These two sugars were found to be contaminated with glucose. Determinations by the glucose-oxidase method, showed that galactose contained about 5 per cent, and maltose about 7 per cent glucose. The contamination with glucose could account for at least part of the enhancing effects of these sugars. However, considering the high glucose content of the maltose preparation, the enhancing effect of this sugar was surprisingly small. We do not know whether this discrepancy, between the glucose content of the maltose and the observed enhancing effect of this disaccharide, can be explained as the consequence of a substrate competition between glucose and maltose in our histamine-release system, or as a result of an unspecificity of the glucose-oxidase method.

B. MESENTERY MAST CELLS

On the assumption that the effect of oxygen deficiency and the action of glucose on the histamine-releasing property of Compound 48/80 reflected a change in the responsiveness of the mast cells, we extended our studies to
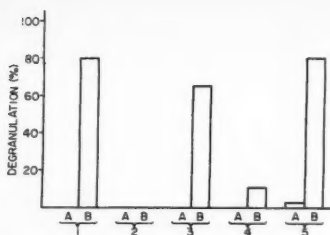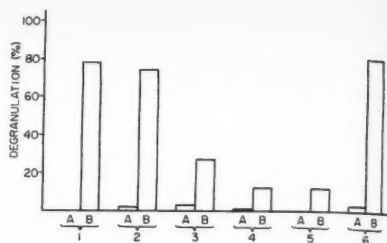
Fig. 9   Fig. 10

Fig. 9. The preservative effect of glucose (5.6 mM) on the mast cell degranulating action of Compound 48/80 (0.5 μg/ml) under nitrogen.
1. 20 min 48/80 (without N_2)
2. 15 min N_2 + 20 min 48/80 N_2
3. 15 min glucose N_2 + 20 min 48/80 N_2
4. 30 min N_2 + 20 min 48/80 N_2
5. 30 min glucose N_2 + 20 min 48/80 N_2
A: Controls in absence of Compound 48/80
B: The effect of Compound 48/80

Fig. 10. The effect of glucose (5.6 mM) on Compound 48/80 (0.5 μg/ml) induced mast cell degranulation after various periods of nitrogenation in absence of glucose.
1. 15 min glucose N_2 + 20 min 48/80 N_2
2. 10 min N_2 + 15 min glucose N_2 + 20 min 48/80 N_2
3. 20 min N_2 + 15 min glucose N_2 + 20 min 48/80 N_2
4. 30 min N_2 + 15 min glucose N_2 + 20 min 48/80 N_2
5. 60 min N_2 + 15 min glucose N_2 + 20 min 48/80 N_2
6. 30 min glucose N_2 + 20 min 48/80 N_2
A: Controls in absence of Compound 48/80
B: The effect of Compound 48/80

include rat mesentery mast cells. Previous failure (Högberg and Uvnäs 1960) to demonstrate an inhibitory effect of anoxia on the degranulating action of Compound 48/80 on mast cells may have been due to inadequate anoxic conditions or to the presence of intracellular energy-yielding substances enabling the degranulation process to develop anaerobically. New attempts were therefore made with cells preincubated in nitrogenated glucose-free medium for various periods. The sensitivity of such cells to Compound 48/80 declined progressively with increasing anoxic preincubation, provided that the subsequent exposure to the releaser was performed under continuous anoxic and glucose-free conditions. Fig. 8 shows 2 exp. in which the mast cells became practically unresponsive to Compound 48/80 within 20—30 min preincubation. There were variations in the length of the anoxic preincubation required to inhibit markedly the mast cell degranulation. In some experiments no preincubation was needed; in others, the inhibitory effect was apparent only after 15—20 min of preincubation.

In the presence of glucose (5.6 mM) the mast cells retained their sensitivity to Compound 48/80, in spite of prolonged exposure to anoxia for at least an hour (15 and 30 min in Fig. 9 and 30 min in Fig. 10).
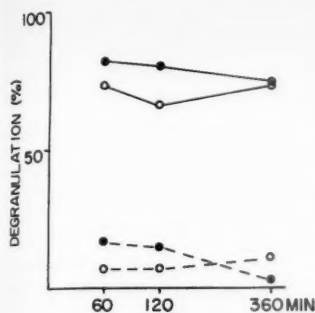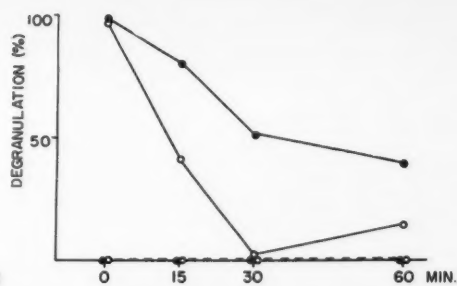
Fig. 11 A and B. The effect of preincubation of rat mesentery pieces, under oxygen (A) and nitrogen (B) in absence of glucose, on the degranulation of mast cells induced by Compound 48/80 (0.5 μg/ml) in glucose-containing (5.6 mM) (•–•–•–•) and glucose-free (o–o–o–o) oxygenated medium. After the preincubation periods the mesentery pieces were incubated a further 15 min under oxygen (in presence or absence of glucose) before the addition of Compound 48/80. Broken lines represent degranulation in absence of Compound 48/80. Abscissa represents preincubation time. Note partial restoration in B on further incubation under oxygen in presence of glucose.
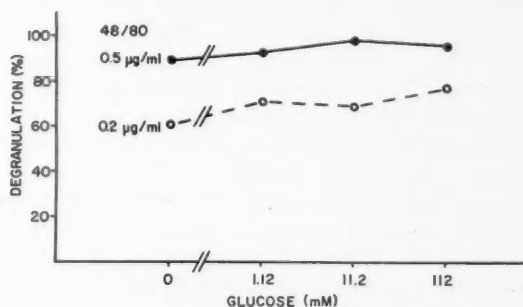
Fig. 12. Mast cell degranulation by Compound 48/80 (0.2 and 0.5 μg/ml) in presence of different concentrations of glucose.

As shown in Fig. 10, in order to exert its preservative action, glucose had to be present from the beginning of the exposure to nitrogen. When added to cells preexposed to nitrogen, glucose progressively lost its restoring effect on the degranulating action of Compound 48/80.

In contrast to the effect of nitrogen, preexposure of mast cells to oxygen for up to 4 hours did not appreciably reduce the sensitivity of the cells. In oxygen, such cells retained their responsiveness, even in the absence of glucose (Fig. 11 A). On the other hand, oxygenation without glucose was unable to restore the inhibitory effect of prolonged nitrogen exposure, while in the presence of glucose a partial recovery occurred, as shown in Fig. 11 B.

Glucose did not enhance the degranulating action of Compound 48/80 under aerobic conditions, as shown in Fig. 12, where glucose concentrations, varying
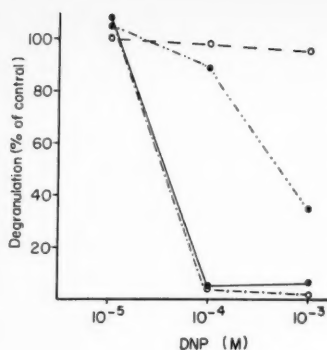
Fig. 13. The effect of glucose (11.2 mM) on the inhibitory action of DNP on degranulation of mast cells by Compound 48/80 (0.5 µg/ml).
•-•-•-•   DNP added 15 min before addition of Compound 48/80 (no glucose present)
o-.o-.o-.o   DNP added 30 min before and glucose 15 min before addition of Compound 48/80
•-..•-..•   DNP and glucose added together 30 min before addition of Compound 48/80
o-o-o-o   Glucose added 30 min before and DNP 15 min before addition of Compound 48/80
All values computed in per cent of the degranulation found in the absence of DNP

from 0 to 112 mM, were without appreciable effect on the degranulation. In this experiment and those subsequently described, the original technique devised by Högberg and Uvnäs was employed.

When mast cell degranulation was blocked by enzyme inhibitors, glucose was found to counteract the blocking effect caused by some metabolic inhibitors which, it is assumed, interfere with oxidative phosphorylation and respiration (DNP, sodium azide, sodium cyanide). For the anti-inhibitory effect to develop fully, the glucose had to be added before the metabolic inhibitor; a fact which is shown in respect of DNP, in Fig. 13. On the other hand, glucose was unable to counteract the blocking action of thyroxin (Table III). The inhibitory action of ninhydrin, allicin, Cu^{++}, Pb^{++} and Zn^{++} — taken as representatives of inhibitors previously shown to block mast cell degranulation — was not influenced by glucose (Table III).

Discussion

The present observations clearly indicate that histamine release from rat lung tissue, as well as degranulation of rat mesentery mast cells, when induced by Compound 48/80, are energy requiring processes. They are both blocked by anoxia and by metabolic inhibitors. The inhibition is counteracted by glucose, indicating that anaerobic pathways are available. The energy-yield from the anaerobic breakdown of glucose seems to offer as favourable conditions for the reactions to proceed as those existing in the presence of oxygen and glucose.

The fact, that histamine release and mast cell degranulation occur under aerobic conditions even when glucose is not added, suggests that also other energy donors are operative.

Table III. The effect of glucose (11.2 mM) on the action of inhibitors of mast cell degranulation induced by Compound 48/80. "With glucose" denotes addition of glucose (11.2 mM) 30 min and inhibitor 15 min before addition of Compound 48/80 (0.5 µg/ml). "Without glucose" denotes addition of inhibitor 15 min before addition of Compound 48/80 (0.5 µg/ml). All values computed in per cent of degranulation in absence of inhibitor

Inhibitor	Concentration of inhibitor (M)					
	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}
Sodium cyanide						
with glucose			105	106	101	93
without glucose			102	101	49	9
Sodium azide						
with glucose			99	92	75	
without glucose			94	11	9	
2,4-dinitrophenol						
with glucose			100	98	95	
without glucose			108	5	7	
Thyroxine						
with glucose			108	47	19[1]	
without glucose			97	26	12[1]	
Ninhydrin						
with glucose			101	22	17	
without glucose			94	10	9	
Allicin						
with glucose	101	52	11	8		
without glucose	91	53	3	3		
Pb						
with glucose	103	89	34	20		
without glucose	106	59	9	9		
Cu						
with glucose	87	65	6	10		
without glucose	104	66	12	3		
Zn						
with glucose		97	70	8	13	
without glucose		104	76	13	8	

[1] Concentration uncertain due to low solubility of thyroxine.

Glucose was able to restore the histamine release inhibited by nitrogenation of the lung tissue, provided that it was added before Compound 48/80 (Table II). Further, in order to counteract the blocking effect of the metabolic inhibitors (DNP, sodium azide, sodium cyanide) on the degranulation of mast cells, glucose had to be added before the inhibitors. Since these inhibitory agents attack the metabolic processes at various points, a reasonable explanation, for the required preincubation with glucose, seems to be, that this substance is utilized by the mast cells, yielding energy through its breakdown.

The inhibition of histamine release from rat lung tissue by DNP was counteracted by glucose, in spite of preincubation with DNP before the addition of glucose. This is in contrast to the results of the corresponding experiments on mast cell degranulation, where glucose was found to have no preservative effect, if DNP was added before the glucose. The reasons for this discrepancy is obscure; but probably it is more of a quantitative than a qualitative nature, and is possibly due to the different experimental conditions.

After preincubation under nitrogen in a glucose-free medium, the restorative effect of glucose on the mast cell degranulation decreased progressively with the incubation time under nitrogen, provided that the subsequent exposure to Compound 48/80 was performed under continuous anoxic conditions. When the mesentery pieces were correspondingly preincubated under nitrogen in a glucose-free medium, a subsequent shift from nitrogen to oxygen did not restore the responsiveness of the mast cells to Compound 48/80. The sensitivity of the mast cells to Compound 48/80 still showed a steep fall with increasing preincubation time under nitrogen. If, however, glucose (5.6 mM) was added at the same time as oxygen was substituted for nitrogen, the mast cell degranulating effect of Compound 48/80 was partly restored. This is in accordance with earlier findings (DIAMANT 1960) on the histamine release induced by Ascaris extract from rat lung tissue, where the inhibitory effect of preincubation under nitrogen in a glucose-free medium was investigated.

DNP is supposed to block, preferably, oxidative phosphorylation. But, as shown in Fig. 6, DNP also blocks histamine release in rat lung tissue under nitrogen, in which histamine release has been restored by glucose, indicating that DNP also inhibits glycolytic energy production. The same inhibition occurs under oxygen (Fig. 6) where DNP-blocked oxydative phosphorylation is counteracted by addition of glucose. Evidently, in these experiments, oxidative phosphorylation is blocked under aerobic conditions by DNP, and under anaerobic conditions by lack of oxygen. When oxidative phosphorylation is blocked in these ways, DNP is still able to counteract the preservative action of glucose on the histamine release, possibly by inducing ATP-ase activity (LARDY and WELLMAN 1953). The inhibitory action of DNP is dependent on the glucose concentration, diminishing with increasing glucose concentration.

The inhibitory action of thyroxin on the mast-cell degranulation was not abolished by glucose. It is obscure whether this difference between thyroxin and the other metabolic inhibitors employed is qualitative or only quantitative.

Since this manuscript was accepted for publication A. M. ROTSCHILD, I. VUGMAN and M. ROCHA e SILVA reported in *Biochem. Pharmacol.* 1961. *7.* 248—256, that histamine release from rat diaphragm *in vitro* induced by Compound 48/80 was blocked by metabolic inhibitors (DNP and NaCN) and by anoxia. The histamine release was partly restored on addition of glucose.

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Aspects of the Glucose and Amino Acid Metabolism in the Liver and the Diaphragm of Normal and Obese-Hyperglycemic Mice

By

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Abstract

Hellman, B., S. Larsson and S. Westman. *Aspects of the glucose and amino acid metabolism in the liver and the diaphragm of normal and obese-hyperglycemic mice*. Acta physiol. scand. 1961. *53*. 330—338. — *In vitro* utilization of uniformly ^{14}C-labelled glucose was studied in the liver and diaphragm in the American variety of the hereditary obese-hyperglycemic syndrome of mice. Only those obese animals were studied, where the livers were without obvious fatty degeneration. There was a higher glucose uptake per liver in the obese-hyperglycemic syndrome. Expressed per unit weight, however, no significant diffences were found either in the liver or in the diaphragm. The same was true for the formation of CO_2 and the incorporation of glucose in the lipid fraction and the insoluble residue after extraction with TCA and alcohol-ether. An incorporation of measurable amounts of carbon atoms from glucose was observed in the following amino acids: in liver, alanine and glutamine, and in the diaphragm, alanine, glutamine and glutamic acid. On a weight to weight basis there were no differences between normal and obese-hyperglycemic mice, except that in the latter the formation of glutamine was 4—5 times higher. The raised conversion of glucose to glutamine in the liver of obese-hyperglycemic mice was the motivation for a separate analysis of the activity of glutamic-oxalacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT), where, however, no statistical differences were observed between the two types of mice.

330

Most experimental work concerning the glucose metabolism in diabetic organisms has been carried out on animals with experimentally produced diabetes. Alloxan has most frequently been used to induce the diabetic state, because injected parenterally it destroys the B-cells in the islets of Langerhans. Since this type of diabetes is caused purely by an insulin lack, it may be supposed to deviate in many respects from the naturally occurring types of diabetes mellitus. It was therefore of great interest when a strain of mice at the Roscoe B. Jackson Memorial Laboratory was found to give rise to offspring with an obese-hyperglycemic syndrome. This syndome is due to the mutation of a single homozygous recessive gene resulting in the expected frequency of 25 % obese offspring (INGALLS, DICKIE and SNELL 1950). In addition to hyperglycemia and obesity these mice are characterized, inter alia, by increased lipogenesis even during fasting (BATES, ZOMZELY and MAYER 1955), hyperphagia (MAYER et al. 1955) and hyperplasia of the islets of Langerhans with greatly increased amounts of depot insulin (WRENSHALL, ANDRUS and MAYER 1955).

The present experiments were performed on obese-hyperglycemic mice and on their lean litter mates, using essentially the same technique as adopted by CHAIN, BELOFF-CHAIN and POCCHIARI (1956) in their series of experiments on alloxan diabetic rats. The utilization of glucose in vitro in the liver and diaphragm was investigated with particular attention to the formation of CO_2 and the incorporation of the glucose in fat and amino acids. The amino acid metabolism in the liver was further studied by determining the activity of two types of transaminases.

Material and Methods

Thirty male obese-hyperglycemic mice (AO-mice) of the American variety (Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine) and 30 of their male lean litter mates (AN-mice) were used for the experiment. The mice, which were more than 6 months old, were allowed free access to food. The AO-mice weighed approximately 50 g, and the AN-mice about 30 g. Only AO-mice which had no macroscopically obvious liver fattening were utilized for the experiment.

Uniformly ^{14}C-labelled glucose was obtained from the Radiochemical Centre, Amersham, England. The radioactive material was diluted to give a specific activity of 2 or 20 μC per mg. Two different incubation media were used:

1. A modified Krebs-Ringer phosphate buffer, pH 7.3, with total glucose 0.0056 M (radioactivity = 0.36 mC/mM). Since, according to KREBS (1950), a high phosphate concentration is incompatible with the physiological concentration of calcium ions, the calcium content was reduced by one third of its original value.

2. A phosphate buffer: NaCl 0.098 M, KCl 0.027 M, $MgSO_4$ 0.0012 M, KH_2PO_4 0.0004 M, Na_2HPO_4 0.0175 M and total glucose 0.0056 M (radioactivity = 3.6 mC/mM) at pH 7.3 (CHAIN et al. 1960).

The animals were killed by cervical dislocation and the tissue samples to be studied were dissected out as quickly as possible. After weighing, the samples were transferred to Warburg vessels, with a total volume of about 5 ml, which contained 0.5 ml of

Table I. μg glucose utilized in different fractions of the liver, expressed per 100 mg wet weight. Glucose concentration of the medium 0.1 %, total radioactivity 1 μC (=160,000 c/min). In the Table are also given the body weights in g and the liver weights in mg (wet and dry weight) and the water content of the liver (%). Mean values ± S.E. The figures within brackets denote the number of animals studied

	Body weight	Liver			μg glucose utilized per 100 mg liver (wet wt.) in different fractions			
		wet weight (mg)	water	dry weight (mg)	TCA	Lipid	Residue	CO₂
AN-mice.. (12)	31.6 ±1.3	1,586 ±93	68.8 ±0.3	495 ±28	68.0 ±2.8	19.2 ±1.9	4.9 ±0.4	5.2 ±0.7
AO-mice.. (12)	48.9 ±1.4	2,393 ±97	66.0 ±0.6	819 ±46	74.8 ±3.8	21.8 ±1.8	5.0 ±0.8	4.8 ±0.5

medium 1 or 2. The liver slices weighed approximately 70 mg. The centre well of the vessels contained small rolls of filter paper, soaked with 30 % NaOH to absorb the respiratory CO_2. Independent of the medium used, the samples were incubated for one hour at 37°, in a gas phase of pure oxygen. After the incubation the Na_2CO_3 formed was collected and precipitated with $BaCl_2$, as described by VILLEE and HASTINGS (1949). The radioactivity was measured as $BaCO_3$. In the incubation experiments the remainder of the liver was used for water and fat determinations. The water content was determined by simply drying the tissue at 40° C until a constant weight was reached. The dried tissue was then extracted in a Soxhlet apparatus with petroleum ether to obtain the fat.

Liver slices from 24 animals were incubated in medium 1. After incubation the liver slices were homogenized and three fractions prepared as described by HELLMAN, LARSSON and WESTMAN (1961). The fractions were: 1) trichloracetic acid (TCA) soluble substances (*e. g.* glycogen and low molecular weight substances such as amino acids and intermediates in carbohydrate metabolism), 2) a lipid fraction and 3) insoluble residue (*e. g.* proteins, nucleoproteins and nucleic acids).

For incubation in medium 2, liver slices and also the diaphragms from 24 animals were used. After one hour of incubation the tissue was treated as earlier described by BELOFF-CHAIN *et al.* (1955). The samples were homogenized and extracted with 60 % ethanol. The dried extracts were diluted to known volumes and transferred to paper for chromatography as described earlier by CHAIN, LARSSON and POCCHIARI (1960). Chromatography was also applied to the medium. All chromatograms were scanned quantitatively by a modification of the automatic device used by FRANK *et al.* (1959). The insoluble residues, left after the extraction with the aqueous alcohol, were treated with 0.05 N NaOH at 40° C for 8 hours and then transferred to planchets and measured for radioactivity.

In addition to the incubations in a glucose-containing medium, the glutamic-oxalacetic (GOT) and the glutamic-pyruvic (GPT) transaminase activities in the liver were determined from a further 12 animals. The tissues were homogenized in 0.1 M phosphate buffer (pH 7.4), and the enzyme activities were determined by the method described by REITMAN and FRANKEL (1957).

Table II. μg glucoce utilized in different fractions of the liver. The figures are calculated on the basis of Table I and give the TCA/100 mg dry wt., lipids/100 mg fat and residue/100 mg fat free dry weight. Mean values ± S.E. The figures within brackets denote the number of animals studied

	TCA/100 mg dry wt.	Lipid/100 mg fat	Residue/100 mg fat free dry wt.
AN-mice.... (12)	218.1 ±9.2	355.0 ±43.5	19.2 ±1.6
AO-mice ... (12)	220.1 ±11.0	366.0 ±26.4	18.5 ±2.5

Results

The values for livers incubated in medium 1 are given in Table I. Both the body weights and the liver weights were considerably higher in the AO-mice. The livers of the latter animals had slightly lower but statistically significantly decreased water content ($t = 4.52$, $P < 0.001$). Calculated per unit wet weight, there were no significant differences between AN- and AO-mice with regard to the radioactivity found in different liver fractions. In Table II the values given in Table I are expressed differently. The radioactivity of the TCA-fraction is given as μg glucose utilized per 100 mg liver dry weight, the lipid fraction as μg glucose utilized per 100 mg fat, and the insoluble residue as μg glucose utilized per 100 mg fat free liver dry weight. Expressed in this way there were still no significant differences between livers from AN- and AO-mice.

Table III gives the results of incubating liver slices in medium 2. As regards the conversion of glucose to CO_2, the values were significantly lower than in medium 1, although there were no differences between the AN- and AO-mice. For the lactic acid in the medium there was no significant difference between

Table III. Utilization of glucose in liver slices from AN- and AO- mice. The results are expressed as μg glucose converted per 100 mg of tissue (wet wt.) after 1 hour of incubation at 37°C in O_2 in 0.5 ml medium. Glucose concentration 0.1 %, total radioactivity 10 μC (=1,600,000 c/min). Mean values ± S.E. The figures within brackets denote the number of animals studied

	CO_2	Lactic acid	Alanine	Glutamine
AN-mice.... (12)	2.55 ±0.19	25.7 ±3.2	0.3 ±0.2	0.6 ±0.2
AO-mice.... (12)	2.84 ±0.38	17.6 ±7.0	0.5 ±0.2	2.5 ±0.6

Table IV. Utilization of glucose in diaphragm from AN- and AO-mice. The results are expressed as µg glucose converted per 100 mg of tissue (wet wt.) after 1 hour of incubation in O_2 in 0.5 ml medium. Glucose concentration 0.1 %, total radioactivity 10 µC (=1,600,000 c/min). Mean values ± S.E. The figures within brackets denote the number of animals studied

	CO_2	Lactic acid	Alanine	Glutamine	Glutamic acid
AN-mice .... (12)	51.0 ±6.3	351.6 ±49.5	4.8 ±0.6	5.5 ±0.5	2.2 ±0.4
AO-mice......... (12)	40.0 ±4.6	297.1 ±28.2	4.2 ±0.6	6.0 ±0.7	2.1 ±0.3

the AN- and AO-mice. The livers were able to convert small amounts of glucose from the incubation fluid into alanine and glutamine. The glutamine formation per unit liver wet weight was 4—5 times greater in the AO-mice. For these mice the quantity of glutamine formed per 100 mg tissue thus corresponded to 2.5 ± 0.6 µg glucose, compared with 0.6 ± 0.2 µg for the AN-mice ($t = 2.99$, $P < 0.01$).

In Table IV the results from the incubation experiments with the diaphragms are listed. It was found that the conversion of glucose to CO_2 and lactic acid was considerably higher in the diaphragm than in the liver. Radioactive alanine, glutamine and glutamic acid were found in extracts from the diaphragm both in AN- and AO-mice. Neither for these amino acids nor for the formation of lactic acid or CO_2 were there any significant differences between the AO- and AN-mice.

As is evident in Table V, there were large individual differences in the activities of glutamic-oxalacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) in the liver. Although the AO-mice showed lower mean values compared with the AN-mice, the differences were not statistically significant.

Table V. Activities of glutamic-oxalacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) in livers from AN- and AO-mice. Values expressed as units per g liver (wet wt.). The range for the determinations are given below the mean values. The figures within brackets in the left column denote the number of animals studied

	$GOT \times 10^4$	$GPT \times 10^4$
AN-mice.......... (6)	3.22 (2.00—4.42)	3.46 (2.74—6.94)
AO-mice.......... (6)	2.78 (2.06—3.28)	3.22 (2.52—4.14)

Discussion

It is evident from previous *in vitro* studies that glucose utilization is reduced in alloxan diabetes. RENOLD *et al.* (1953) and RENOLD, HASTINGS and NESBETT (1954) thus showed that the glucose uptake and phosphorylation is lower in liver slices from alloxan diabetic rats. A corresponding decrease of the glucose uptake was also found in the skeletal musculature (VILLEE and HASTINGS 1949 and BEATTY *et al.* 1959). In view of these observations of a decreased glucose utilization in alloxan diabetes, it is therefore of interest that in the present experiment no significant differences were recorded regarding the glucose utilization per unit weight either for the liver or the diaphragm in the obese-hyperglycemic syndrome.

In the case of the liver this was true for the different fractions analyzed, regardless of whether the values were calculated per unit wet or dry weight, or whether the fat content of the liver was taken into consideration. On the other hand it is also worthy of note that there was, *in vitro*, no increase in incorporation in the glycogen-containing TCA fraction, since SHULL and MAYER (1956) reported, after intraperitoneal administration of uniformly ^{14}C-labelled glucose, that the turnover of liver glycogen was approximately 3 times as great per unit liver weight in the AO-mice, in spite of the fact that the glycogen concentration was the same in the two groups. The absence of any differences in the glucose utilization per unit weight of liver and diaphragm in our experiments should further be considered with reference to the condition, that the glucose concentration in the incubation medium was the same for both groups and thus considerably less than the blood sugar values for obese-hyperglycemic mice with free access to food (MAYER, BATES and DICKIE 1951).

While a raised glycogen content of skeletal muscle has been reported in the obese-hyperglycemic syndrome (SHULL and MAYER 1956), there was no previous data about the *in vitro* glucose utilization of the diaphragm in AO-mice. It is of interest with regard to the absence of a significant decrease in the glucose uptake by the diaphragm of the AO-mice that BELOFF-CHAIN *et al.* (1955) did not either find any changes in rats, which had a less advanced degree of alloxan diabetes. In assessing the results, it should furthermore be recalled that they do not directly reflect the true rates of oxidation, since a considerable and quantitatively undetermined dilution of the isotope takes place during the incubation, primarily through glycogenolysis and gluconeogenesis.

The synthesis of lipids constitutes an important part of liver function. The first indication that this was disturbed in alloxan diabetes was found by STETTEN and BOXER (1944), who observed during *in vivo* experiments an almost complete inhibition of fatty acid synthesis from glucose in the liver of alloxan diabetic rats. These results were confirmed and extended by CHERNICK and CHAIKOFF (1950); CHERNICK *et al.* (1950) by *in vitro* experiments with [14]C-

labelled compounds. According to these authors, in alloxan diabetes the synthesis of fatty acids from glucose is reduced more than, for example, the oxidation of the glucose to CO_2. The available information concerning lipid metabolism in the obese-hyperglycemic syndrome is mainly based on *in vivo* studies of the incorporation of C-2-fragments, the condensation of which is a stage in fatty acid synthesis. BATES, MAYER and NAUSS (1955) showed that when ^{14}C-carboxyl-labelled acetate was fed to the AO-mice, these retained significantly more radioactivity in the liver and carcass fat. On the basis of similar experiments with the same precursor, GUGGENHEIM and MAYER (1952) suggested, that a partial block of the C-2-fragment oxidation was the characteristic biochemical lesion in the obese-hyperglycemic syndrome. While PARSON and CRISPELL (1955) did not confirm this, HUGHES and TOLBERT (1958), after intraperitoneal and intravenous injections of ^{14}C-methyl-labelled acetate, found that both the rate and cumulative excretion of [14]CO_2 from the acetate were depressed in the AO-mice.

Of further interest is the report of greater hepatic lipogenesis in the AO-mice, both per unit weight of liver and fat free tissue, after incubating liver slices with ^{14}C-carboxyl-labelled acetate (MAYER *et al.* 1955). It is hardly possible to make any direct comparison between these experiments and our studies of lipogenesis, since MAYER *et al.* did not add glucose to the medium. As shown by MILSTEIN and HAUSBERGER (1956), there is a particularly marked reduction in hepatic lipogenesis upon lowering the glucose concentration in the medium. With regard to our finding that in the AO-mice there were differences for the conversion of glucose to fat only in the case of the whole liver, and not per unit weight or in "specific activity", this may perhaps have some connexion with the fact that we used only AO-mice, which had no macroscopically obvious signs of liver fattening. The absence of real fatty degeneration in the livers of the AO-mice is supported not only by the fat analysis but also by the water content values, where the differences between the two groups, although statistically significant, were relatively small (*cf.* FINLAYSON, KROOK and LARSSON 1960).

It is known that protein synthesis is subnormal with insulin deficiency but may be restored to normal by insulin administration. Insulin alone, added *in vitro* to liver slices from mildly diabetic rats, does not stimulate amino acid incorporation, whereas glucose alone does, and insulin and glucose together have a still greater effect (KRAHL 1960). The existence of a simultaneous hyperglycemia and hyperinsulinism in the AO-mice made it especially interesting to study their protein metabolism as reflected in the formation of amino acids from glucose. After incubation in uniformly labelled ^{14}C-glucose radioactivity was found, in liver, in alanine and glutamine, and, in the diaphragm, in alanine, glutamine and glutamic acid, for both the AN- and the AO-mice., It was characteristic of the amino acid pattern in the obese-hyperglycemic syndrome, that the rate of formation of glutamine per unit

liver wet weight was 4—5 times greater. The biological importance of this observation is difficult to evaluate. The presence of the enzyme glutamotransferase and reports that the plasma level of glutamine increases with a positive nitrogen balance have been considered as indicative of an essential role of glutamine in protein metabolism (WHITE *et al.* 1954). Since glutamine is easily hydrolyzed to the metabolically active glutamic acid, comparative studies of the reactions in which glutamic acid take part are of great interest. In the present investigation, therefore, the transamination process was taken up as a separate study, in which, however, no definite differences were observed between the AO- and the AN-mice with respect to the activity per unit liver weight of glutamic-oxalacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT).

The choice of incubation medium significantly influenced the rate of formation of radioactive CO_2 in the liver slices. The formation of labelled CO_2 was thus significantly lower when medium 2, containing, *inter alia*, more K^+ than medium 1, was used. It may be mentioned that the content of K^+ has been previously shown to have considerable effect on the CO_2 production from uniformly ^{14}C-labelled glucose in the brain tissue (CHAIN, LARSSON and POCCHIARI 1960).

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A Comparison of the Distribution of Radioactive Fluorine and Calcium by Use of Double-Isotope Autoradiography

By

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Abstract

Appelgren, L.-E., Y. Ericsson and S. Ullberg. *A comparison of the distribution of radioactive fluorine and calcium by use of double-isotope autoradiography.* Acta physiol. scand. 1961. *53*. 339—347. — The autoradiographic distribution picture has been compared in whole pregnant mice and young rats at short intervals after the injection of fluorine (NaF^{18}) and calcium (Ca^{45}Cl$_2$). The isotopes were injected intravenously on the same animal and selective registration of F^{18} and Ca^{45} was made utilizing the great difference in decay rates. Both isotopes, especially Ca^{45}, were rapidly taken up from the blood. F^{18} accumulated more rapidly and selectively in hard tissues while Ca^{45} was taken up in appreciable amounts in some soft tissues. The distribution pattern in bone and in dentine was very similar for F^{18} and Ca^{45}. F^{18} was taken up in proportionately low concentration in the calcifying molar enamel. Ca^{45} but not F^{18} was taken up in the cartilage of young animals.

A remarkably high accumulation of Ca^{45} was seen in some groups of striated muscles. These muscle groups may be those which were contracted when the lightly anesthetized animal was sacrificed by immersion in the freezing mixture. The dominating excretory organ for F^{18} was the kidney while high concentrations of Ca^{45} appeared in the mucosa and contents of the small intestine.

The role of fluorine in general physiology and caries prevention is essentially dependent on the specific uptake of fluoride ions by the calcified tissues of the body. Rather farreaching similarities have been noticed in the patterns of uptake of F^{18} and Ca^{45} in hard tissues. It is generally considered that the uptake of F^{18} in the skeleton is associated with an incorporation in the hydroxy apatite crystals, probably through an exchange with OH-ions.

In the present work we have compared the autoradiographic distribution picture in sections through whole pregnant mice and young rats at short intervals after the injection of NaF^{18} and $Ca^{45}Cl_2$.

In order to eliminate disturbing influence from biological variation the distribution of both isotopes has been studied on the same animal — a procedure which does not seem to have been utilized previously and which we have termed double isotope autoradiography.

The widely diverse decay rates (half life of F^{18} 110 min and of Ca^{45} 153 days) form the basis of selective registration of the two isotopes. With appropriate dosage of F^{18} and Ca^{45} the initially dominating F^{18} radiation can be registered during a few hours without detectable interference from Ca^{45}. After the decay of F^{18} the Ca^{45} radiation is registered on a second film during some weeks' exposure.

Methods

Radioactive fluoride solutions were prepared according to methods described earlier (ERICSSON and ULLBERG 1958, ERICSSON, ULLBERG and APPELGREN 1960). These methods include the production of F^{18} by neutron irradiation of lithium hydroxide, purification of F^{18} by distillation and concentration of the activity by evaporation of the distillate.

$Ca^{45}Cl_2$ (spec. act. 1. 74 C/g) was obtained from O. R. N. L., Oak Ridge, Tenn., USA.

Three albino mice in advanced pregnancy (two days before expected parturition) and two 15-day-old albino rats were used in the experiments. The radioisotopes were administered by injection in a tail vein. The radiofluoride solution was immediately followed by that containing the radiocalcium. The F^{18} dose was about $2 \mu C$ per g body weight and the Ca^{45} dose about $0.05 \mu C$ per g body weight.

The time interval between injection and sacrifice was 2 min for one of the pregnant mice and for other animals 30 min. The slightly ether anaesthetized animals were sacrificed by immersion in acetone with solid CO_2 added, the temperature being about $—78°$ C.

The frozen animals were sectioned in a refrigerated room at $—10°$ C. Sagittal 20μ-sections were cut through the whole animals. Some of the sections were rapidly freeze-dried in a modified Glick-Malmström apparatus before the autoradiographic exposure. However, in the majority of cases undried sections were used, chemical fogging being avoided by making the exposure at a lower temperature in boxes half-filled with CO_2-snow.

The autoradiography was largely made according to methods described earlier (ULLBERG 1958, ERICSSON and ULLBERG 1958). The films used were Structurix (Gevaert) and Kodirex (Kodak). The exposure time in the case of registration of F^{18} was 3 hours. After a delay of 2 days in order to allow the F^{18} to decay a new exposure was made, which gave Ca^{45} autoradiograms after 3—5 weeks.

A 4-hour control exposure, after the decay of F^{18}, gave no detectable autoradiographic blackening, which proved that the Ca^{45} did not interfere noticeably during the registration of F^{18}.

Results

Fig. 1—4 show the positives of representative autoradiograms. Owing to the lower radiation energy of Ca^{45} the resolution of the Ca^{45} autoradiograms is better than that of the F^{18} autoradiograms.
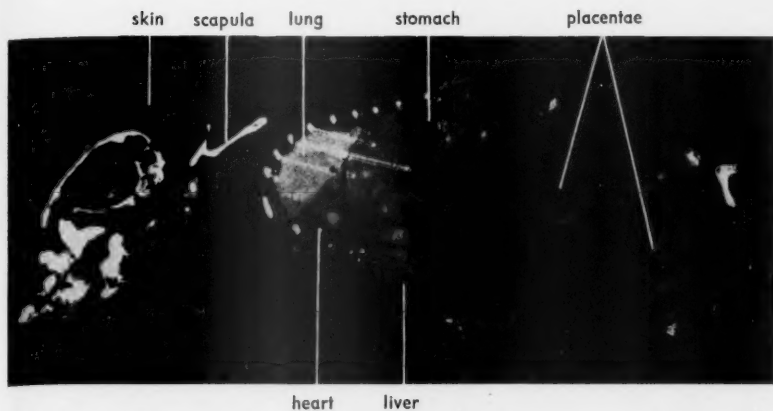
Fig. 1 a. Autoradiogram showing the distribution of F^{18} in a pregnant mouse, 2 minutes after intravenous injection of NaF^{18} + Ca^{45}Cl$_2$. The white areas correspond to high fluorine content. A heavy accumulation in bone can already be seen.
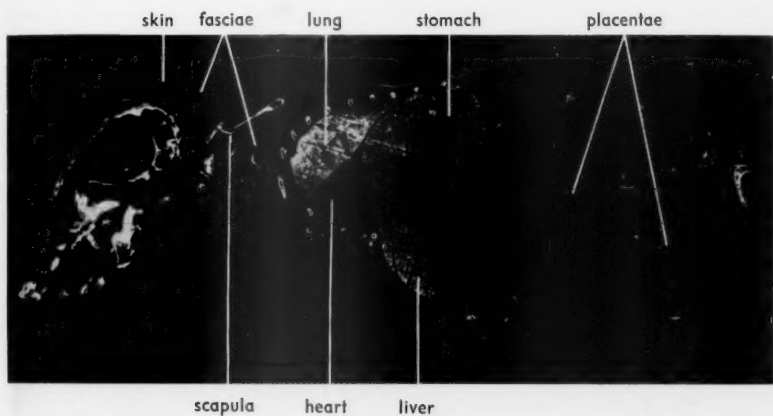

Fig. 1 b. Autoradiogram from the same section as in Fig. 1 a, showing the distribution of Ca^{45}. Note accumulation in bone, lung, muscle fasciae and liver.

Two minutes after the intravenous administration of the two isotopes the blood concentration of both F^{18} and Ca^{45} is still fairly high. (Fig. 1)

F^{18} seems to have been taken up relatively more rapidly in the hard tissues, while Ca^{45} is seen in rather high concentration in some soft tissues. The concentration of radiocalcium in muscle fasciae, lung, skin, and liver is considerably higher than in the blood. None of the isotopes occurs in registrable amounts in the central nervous system and the foetuses show very little uptake.
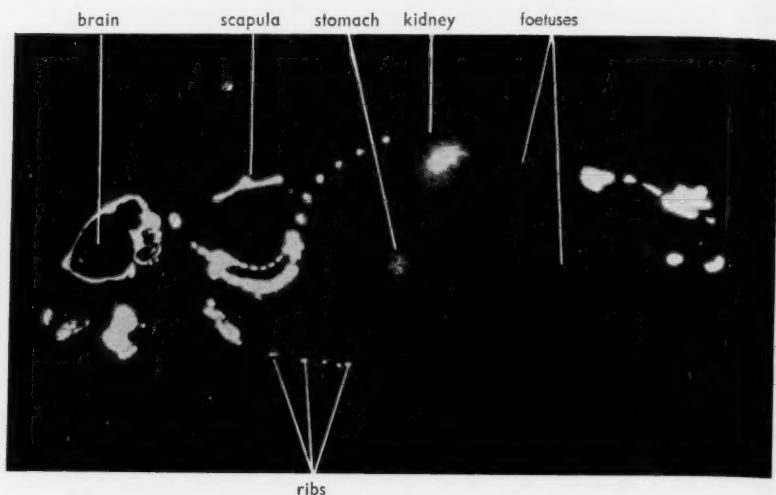
Fig. 2 a. Autoradiogram of F^{18} in a pregnant mouse, 30 minutes after intravenous injection of $F^{18} + Ca^{45}$. The radiofluorine is accumulated selectively in the hard tissues. High concentration is also seen in the renal medulla.
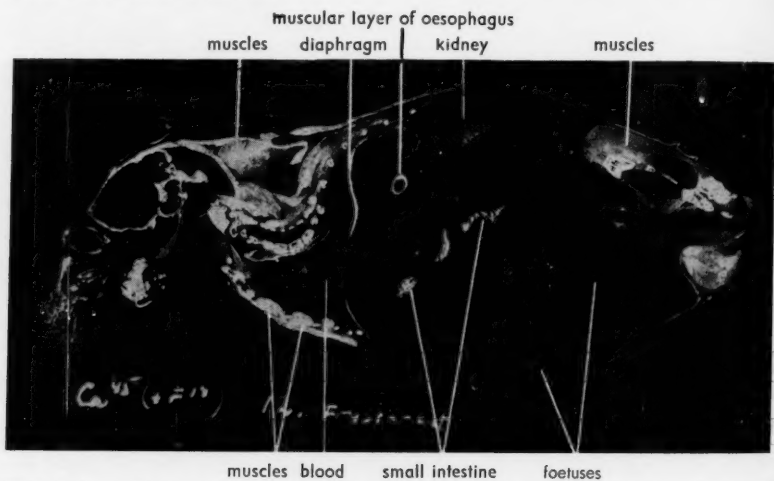
Fig. 2 b. Autoradiogram of Ca^{45} from same section as 2 a. The Ca^{45} is cleared from the blood. Accumulation of Ca^{45} is seen in the hard tissues but also in some muscle groups and in the mucosa and contents of the small intestine.

Fig. 2 shows the distribution in a pregnant female 30 min after intravenous administration. Striking differences now appear in the distribution patterns of F^{18} and Ca^{45}.

F^{18} has accumulated selectively in the hard tissues, which show a very high concentration. All soft tissues show a low and even concentration of F^{18} except the renal medulla where an accumulation can be seen, obviously in connection with excretion of part of the injected radiofluorine. (The presence of F^{18} in the gastric lumen, which has never been noticed in our previous F^{18} studies, is probably due to the fact that the mouse has lapped up some urine or urine-contaminated material.) The blood shows about the same F^{18} concentration as most of the soft tissues.

The Ca^{45} seems to have left the blood almost totally. It is, however, not at all as selectively located in the hard tissues as is F^{18}. An appreciable concentration of Ca^{45} can be noticed in some groups of striated muscles. Thus a very high concentration of Ca^{45} can be seen in the muscular part of the diaphragm, while the tendinous part does not show noticeable quantities of Ca^{45}. A high concentration of Ca^{45} has been found in the myocardium on other sections.

High concentration of Ca^{45} is seen in some muscle groups in the sternal, neck, and pelvic regions and in some facial muscles like the muscles of the eye and the tactile hairs. In some autoradiograms it is evident that muscle bundles running in one direction show high Ca^{45} uptake while other muscle bundles, crossing the first-mentioned, do not show noticeable concentration of Ca^{45}. The muscular layer of the oesophagus also shows high Ca^{45} concentration while the concentration in the intestinal muscles is low.

The excretion picture is also very different. While the F^{18} is excreted mainly in the kidney the Ca^{45} concentration is relatively low in the kidney but high in the intestine, especially in some loops of the small intestine. The liver shows a lobular pattern in the Ca^{45} autoradiograms and signs of a slight excretion of Ca^{45} via the biliary ducts are seen.

The central nervous system after 30 minutes still shows very low concentration of both F^{18} and Ca^{45}. Signs of accumulation of Ca^{45} are, however, seen in the choroid plexus. The foetuses show a selective uptake of both F^{18} and Ca^{45} in the mineralised hard tissue portions.

Another pregnant mouse, killed 30 minutes after the injection, showed a very similar distribution of both F^{18} and Ca^{45}. Thus high concentration of Ca^{45} was found in the diaphragm, myocardium, and in about the same groups of skeletal muscles as in the aforementioned case.

In Fig. 3 the distribution of F^{18} and Ca^{45} is compared in a 15-day old rat, killed 30 min after intravenous injection.

The F^{18} has as usual accumulated selectively in the hard tissues and is also seen in the kidney.

A clear difference can be noted in the distribution pattern in the teeth (see enlargement Fig. 4). The F^{18} uptake in the molar enamel is very slight in comparison with the dentine, while the relative enamel uptake of Ca^{45} is considerably higher. This does not, however, come out with the unerupted upper incisor.
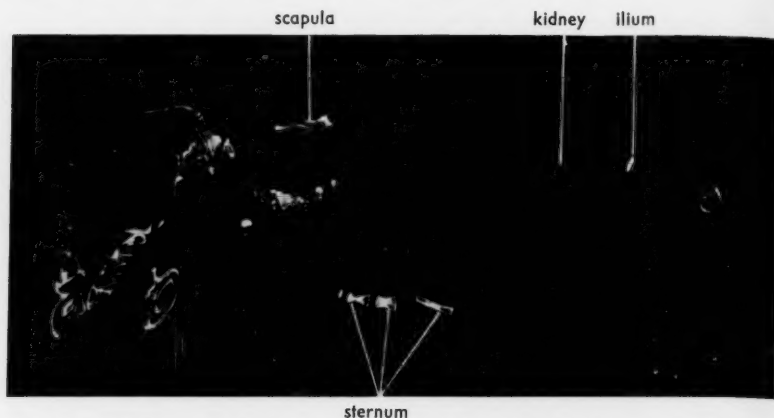
Fig. 3 a. Autoradiogram of F^{18} in a 15 days old rat, 30 minutes after intravenous injection of $F^{18} + Ca^{45}$. Accumulation of F^{18} can be seen only in the hard tissues and (due to the excretion) in the kidney.
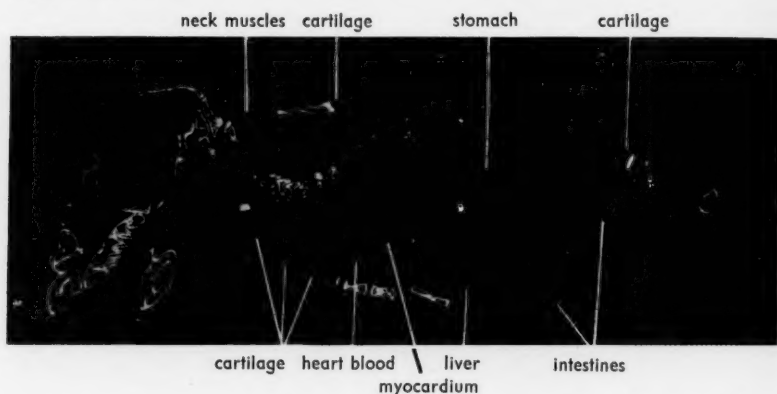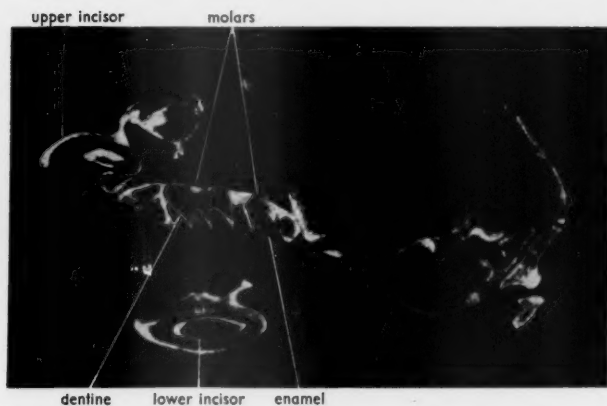

Fig. 3 b. Autoradiogram of Ca^{45} from the same section as 3 a. No Ca^{45} can be seen in the blood. The highest uptake can be noted in the hard tissues, but a fairly high concentration can also be seen in cartilage, some muscles, liver and intestinal mucosa and contents.

In the sternal and rib cartilage an uptake of Ca^{45} is seen, which is lower than that of bone but still marked. In the F^{18} autoradiogram no corresponding uptake can be noticed. A similar difference can be seen in the not yet ossified cartilage of the pelvic bone and scapula. A fairly high concentration is also seen in some muscles in the neck region and in the myocardium, but the diaphragm in this case shows a low level.

A high concentration of Ca^{45} is seen in the liver. The intestine, especially the small intestine, shows an accumulation of Ca^{45} in its mucosa and contents.

Fig. 4 a. Autoradiogram of F¹⁸ from the head of a 15 days old rat, 30 minutes after intravenous injection of F¹⁸ + Ca⁴⁵. Note low uptake in the enamel of the upper molars.
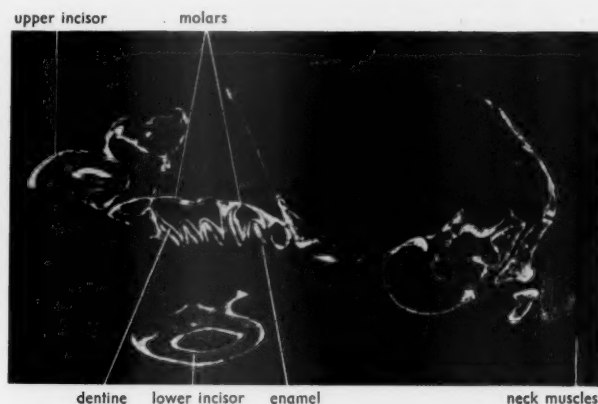
Fig. 4 b. Autoradiogram of Ca⁴⁵ from the same section as 4 a. Note high uptake in the enamel of the upper molars.

Discussion

In the above description of results the basis of comparison between F¹⁸ and Ca⁴⁵ distributions has been the relative filmblackening by different tissues. The radiation absorption within the sections has been negligible for both F¹⁸ and Ca⁴⁵ and therefore has not caused appreciable alterations of the relative intensity of radiation among different tissues.

Concerning the distribution in hard tissues the observation of a relatively low uptake of F^{18} in the molar enamel agrees with the low concentration of fluorine found in chemical analysis of the enamel as compared with the dentine (review: BREDEMANN 1956).

The inference of the observation that Ca^{45} but not F^{18} accumulates in cartilage in young animals may perhaps be that the calcium is not yet incorporated in apatite crystals, which may be a pre-requisite for the uptake of fluorine. This may be of importance for estimating the role of high doses of fluorine in sclerotization of cartilage and ligaments and deserves further study.

In bone, however, F^{18} and Ca^{45} show a more coinciding distribution pattern. It is evident that differences in distribution concern especially the soft tissues.

The most astonishing finding is the high concentration of Ca^{45} in certain muscle groups, especially in view of the low total calcium content found in muscle tissue (EVERETT 1946, GROSSE-BROCKHOFF 1950, BRONNER 1958). As the lightly anaesthetized animals made warding-off movements at immersion in the freezing mixture it is conceivable that the muscles which were contracted in the freezing moment may be the ones that show high Ca^{45} content in the autoradiograms.

The finding of Ca^{45}-negative muscle bundles crossing Ca^{45}-rich bundles may support this theory. The observation may perhaps be put in relation to the finding made in investigations with isolated muscles showing that the strength of a muscle contraction depends on the calcium ion concentration and that a very low calcium concentration completely prevents contraction (PAUSCHINGER and BRECHT 1961).

The possibility that the distribution of Ca^{45} 30 min after intravenous injection is not representative for the distribution of total calcium must be considered. In this connection a recent finding of LIKINS and CRAVEN (1960) may be mentioned. Starved rats excreted much less of injected Ca^{45} through the kidneys than normally fed rats in spite of an equally great urinary excretion of chemically analyzed calcium among the two groups of animals. These authors suggested an uneven partitioning of the injected label among the different calcium pools.

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Osmotic Behaviour of the Epithelial Cells of Frog Skin

By

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Abstract

MacRobbie, E. A. C. and H. H. Ussing. *Osmotic behaviour of the epithelial cells of frog skin.* Acta physiol. scand. 1961. *53.* 348—365. — The osmotic behaviour of the frog skin epithelium has been investigated by microscopic measurements of volume under different experimental conditions. Simultaneous measurements of potential and short-circuit current were made. The effects of changes in tonicity of both bathing solutions and of ionic replacements (K^+ for Na^+ $SO_4^=$ for Cl^-) were studied. Furthermore the active sodium transport was stimulated by short-circuiting and by application of antidiuretic hormone, and inhibited by low pH and by g-strophanthin.

The following conclusions could be drawn:

1) The outward facing boundary of the epithelium is permeable to Na^+ and Cl^-, but impermeable to K^+ and $SO_4^=$.

2) The inward facing boundary is permeable to K^+ and Cl^- but practically impermeable to Na^+ and $SO_4^=$.

3) The outward facing boundary is much less permeable to water than is the inward facing one.

4) Application of antidiuretic hormone to the inside bathing solution increases the water permeability of the outward facing boundary whereas the inward facing membrane is unaffected.

5) Inhibition of the active sodium transport by either g-strophanthin or by low pH in the inside bathing solution was accompanied by a pronounced decrease in the passive ion permeabilities.

6) The results strongly indicate that potassium is transported actively from the inside bathing solution into the epithelium.

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348

Table I. *Ion concentrations in the Ringer's solutions used (mM)*

	Chloride RINGER	Sulphate RINGER
Na$^+$	113.5	113.5
K$^+$	1.88	5.0
Ca^{++}	1.08	1.08
Cl$^-$	115	0
HCO$_3^-$	2.4	2.4
SO$_4^=$	0	58.9

Previous studies have indicated (KOEFOED-JOHNSEN and USSING 1958) that the electric potential across the frog skin can be satisfactorily explained on the basis of the following assumptions:

1) The outward facing boundary of the epithelium is permeable to Cl$^-$ and a few other small anions (Br$^-$, HCO$_3^-$) but virtually impermeable to larger anions. It is selectively, but passively, permeable to Na$^+$ and Li$^+$, whereas K$^+$ and all other cations permeate extremely slowly if at all.

2) The inward facing membrane is permeable to K$^+$ and Cl$^-$ (and a few other small ions like Rb$^+$, NH$_4^+$, HCO$_3^-$, Br$^-$ etc.) but impermeable to large ions including Na$^+$, Li$^+$ and SO$_4^=$.

3) The inward facing membrane is provided with a "sodium pump" which extrudes sodium from the cells, probably in exchange for K$^+$.

Whereas the existence of the active sodium transport — the "sodium pump" — has been well established (for references see USSING and ZERAHN 1951) it was felt that the specific permeability properties postulated above ought to be verified by observations which are independent of the potential measurements. It seemed likely that a study of the volume changes of the epithelium in response to changes in the composition of the two bathing solutions could provide the desired information about the ionic selectivities of the two epithelial boundaries. Thus, if the inward facing membrane is permeable to KCl but impermeable to NaCl, replacement of the Na$^+$ of the inside solution by K$^+$ should lead to diffusion of KCl into the cells and thus to osmotic swelling. The same procedure applied to the outside solution should lead to a slight shrinkage if it is true that the outer membrane is permeable to Na$^+$ but not to K$^+$.

Therefore the method described below was developed, permitting accurate microscopic measurement of the volume of the frog skin epithelium under different experimental conditions including changes in tonicity of the bathing solutions as well as ionic replacements (K$^+$ for Na$^+$, SO$_4^=$ for Cl$^-$ etc.). In addition treatments known to influence the active sodium transport were studied for their effect on cell volume. Thus the sodium transport was enhanced by short-circuiting (USSING and ZERAHN 1951) and by treatment with antidiuretic
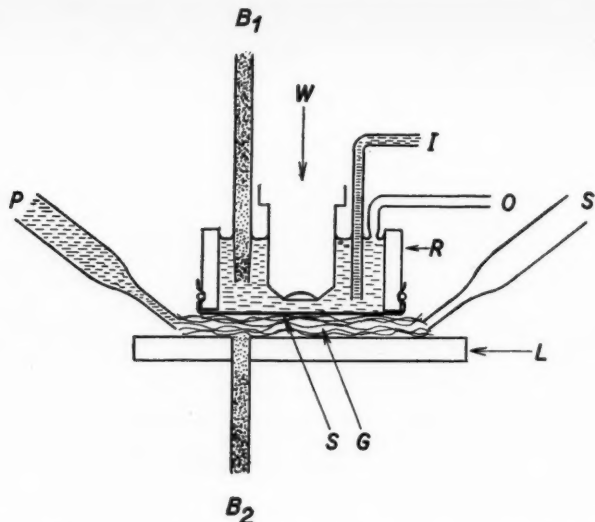
Fig. 1. Diagram of the apparatus for measuring changes in the thickness of the frog skin epithelium.

B_1 and B_2 3 M KCl-agar bridges.
S   frog skin surface, inside.
G   glass wool pad.
L   lucite plate.
R   plastic ring.
W   water immersion lens of the microscope.
P   inflow pipette for "inside bathing fluid".
S   outflow pipette, connected to suction flask.
I   inflow of "outside bathing fluid".
O   outflow of "outside bathing fluid".

hormone (FUHRMAN and USSING 1951), and inhibited by low pH (USSING 1949, SCHOFFENIELS 1956) and by treatment with g-strophanthin (KOEFOED-JOHNSEN 1957).

The experimental evidence concerning the ion permeabilities obtained in these studies closely parallels that found by electrical measurements.

Methods

The experiments were done mainly on the abdominal skin of the brown frog, *Rana temporaria*. The frog were pithed and the skin was then removed and washed in the appropriate Ringer's solution (chloride or sulphate Ringer). The composition of the Ringer's solutions used is given in Table I.

The skin was mounted on the microscope stage as shown in Fig. 1, tied by a cotton thread on a grooved plastic ring (diameter 3 cm, height 1.5 cm), with the outside of the skin facing upwards. The ring was then placed with the inside of the skin in con-
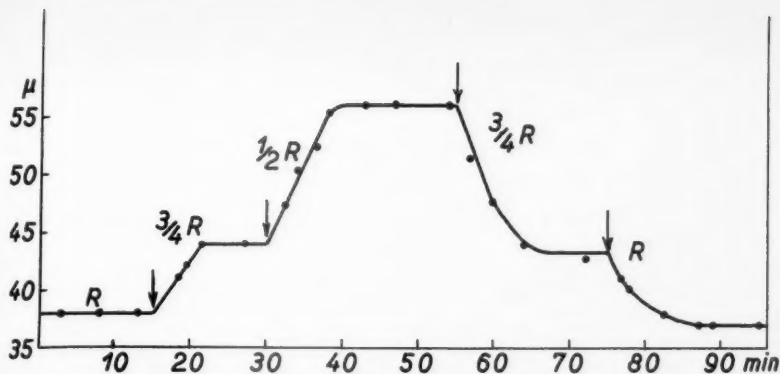
Fig. 2. Volume changes associated with tonicity changes of the inside solution: sulphate Ringer. The outside medium is distilled water throughout the experiment.

The total measured thickness of the epithelium in microns is plotted against time in minutes, for various dilutions of sulphate Ringer (R, 3/4 R, 1/2 R).

tact with a thin glass wool pad on a shallow lucite dish. A regular flow of solution through the glass wool pad bathed the inside of the skin and a flow was also maintained through the chamber formed by the outside of the skin and the wall of the plastic ring; this allowed rapid changes of the solution bathing both sides of the skin. Volume measurements were made microscopically. The skin was observed through a Leitz water immersion lens in the outside bathing solution (objective magnification × 50, eyepiece magnification × 12.5). The objective was electrically insulated from the body of the microscope by a plastic ring. The vertical distance between two reference points, one on the outside surface of the skin and the other on a pigment cell immediately below the epithelial cell layer, was measured using the calibrated finefocussing control of the microscope. By carefully choosing a spot where a sharp line on the surface coincided with a sharp outline of a melanophore cell underneath, it was possible to determine this vertical distance between the two levels with an accuracy of about 1 μ.

Under the conditions of the experiments the area of the skin (and thus of the epithelium) remains constant. Therefore any change in the volume of the epithelium becomes proportional to the change in height. Lateral movements within the observed area are usually nil or quite insignificant, even during gross changes in the cellular volume.

The potential across the skin was recorded continuously during experiments by means of an automatic balancing apparatus constructed and described by MULLINS (1958), this was calibrated by measuring the potential with a high impedance, null-reading millivoltmeter (PHM 3, Radiometer, Copenhagen). The potential measuring electrode in the inside solution consisted of a 3 M KCl-agar bridge set in the lucite dish, connecting the glass wool pad with a Ringer-filled vessel cemented to the under-side of the lucite dish, and connected in turn by a 3 M KCl-agar bridge to a calomel half-cell. A fine-tipped 3 M KCl-agar bridge set in the outside bathing solution was connected to a second calomel half-cell and served as the other potential electrode. In this arrangement relatively low electrode resistances, for use with the recorder (input impedance 50 kΩ), were obtained without excessive diffusion of KCl from the agar bridges into the bathing solutions.

Table II

	V_r (μ)	V_o (μ)	V_i (μ)	Water permeability of inner membrane (10^{-4} cm/sec.)
Range............	$35 - 86$	$12 - 31$	$16 - 64$	$15 - 34$
Mean .... (with S. E. and no. of skins in brackets)	58 ± 3 (25)	21 ± 1 (25)	37 ± 2 (25)	24 ± 1 (28)

It was possible to short-circuit the skin in this set-up. The recorder was then used as described by MULLINS, both to maintain the skin with zero potential across it and to record the current necessary to do this. The current electrodes (not shown in Fig. 1) consisted of two platinum wires, one dipping into the outside solution and the other set under the glass wool in a tortuous groove in the lucite plate, arranged to give as uniform coverage of the skin as possible. The variation of the shortcircuit current with the position of the outside potential electrode was, at worst, 5 per cent so that the field over the skin seemed to be reasonably uniform, in spite of the acentric positions in the outside solution of both the outside current electrode and a large conducting body, the microscope objective at its working distance of 0.44 mm. Also the conductivity of Ringer's solution is high enough for the skin to be short-circuited even directly under the non-conducting lens (2 mm diameter) of the objective.

After mounting the skin, volume and potential measurements were made for 1—2 hours until the skin had reached a steady state. Only when this level had been firmly established were the experimental conditions changed and the consequent volume and potential changes followed.

Results

Changes in the tonicity of the inside bathing solution: sulphate Ringer

With sulphate Ringer inside and sulphate Ringer or distilled water outside, changes in the tonicity of the sulphate Ringer bathing the inside of the skin produced regular and reproducible volume changes in the epithelial cell layer. A typical curve of volume against time in such an experiment is shown in Fig. 2. On dilution of the inner solution the cells swelled to a new steady level, and shrank again on return to a more concentrated solution. Judging from the reversibility of the volume changes no solute enters or leaves the cells in the sulphate medium. A slight shift downwards of the steady state level may be seen during the first swelling and shrinking cycle induced in a skin, as if some material were irreversibly lost, but thereafter only water seems to pass the membrane, no matter how often the process of changing the osmotic pressure is repeated. Using the thickness of the epithelium as a measure of the epithelial volume the observations demonstrated that the fractional changes in the latter were considerably smaller than the fractional changes in the tonicity, indicating that only some part of the total epithelial volume participated in the volume
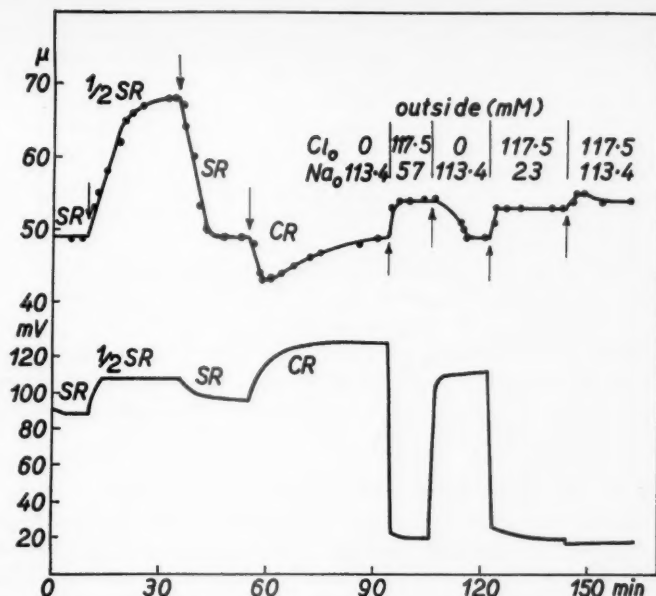
Fig. 3. Volume changes and changes in skin potential associated with changes in the bathing solutions.

Ordinate, upper curve: thickness of epithelium in micra, lower curve: skin potential in millivolts (inside positive).

Abscissa: time in minutes. In the first 95 minutes the outside medium is continuously sulphate Ringer whereas the composition of the inside medium is varied:

First Sulphate Ringer (SR) and then in succession 1/2 sulphate Ringer (1/2 SR), sulphate Ringer and chloride Ringer (CR). After 95 minutes the inside medium is chloride Ringer throughout, whereas the outside medium is varied: first modified chloride Ringer with half the Na$^+$ replaced by potassium, then sulphate Ringer, chloride Ringer with 4/5 of the Na$^+$ replaced by potassium and finally ordinary chloride Ringer.

changes, whereas the remainder was osmometrically inert. Calling the thickness equivalent of the non-osmotic volume V_i, the thickness equivalent of the osmotic volume V_o and the measured thickness V_r the experimental data were found to fit the equation:

$$V_r - V_i = \text{constant/tonicity}$$

from which the thickness equivalents of non-osmotic and osmotic volumes could be calculated. This relation was shown to hold in solutions of tonicities relative to sulphate Ringer of 1, 1.35, 2, 0.50, 0.67 and 0.75. The rates of swelling and shrinking in any of these solutions appeared to be governed by a constant permeability factor, independent of cell volume. The water permeability of the inner membrane was calculated as the water flux (moles cm^{-2} sec^{-1}) per unit water

Table III

Inside solution	Outside chlcride (mM)	Osmotic volume (μ) $\sim V_o$		Cell chloride (mM)	
		Range	Mean	Range	Mean
RINGER	117.5	21 — 38	27 ± 2 (6)	44 — 53	49 ± 2 (6)
RINGER	0	13 — 26	21 ± 1.5 (10)	20 — 41	31 ± 2 (10)

concentration difference (moles cm^{-2}). The above relation between cell volume and tonicity justifies two assumptions on which the interpretation of later results is based, (1) that changes in volume give rise to changes in height of the cells only, without any lateral expansion, and (2) that the volume changes are not restricted by elasticity of the cell. Table II summarizes the results obtained in these experiments; V_o is the thickness equivalent of the osmotic volume in skins pre-equilibrated in sulphate Ringer.

Changes in the tonicity of the outside bathing solution: sulphate Ringer

The volume of the epithelial cell layer in sulphate Ringer solutions appeared to be independent of the tonicity of the outside bathing solution; the volume remained constant whether this was normal sulphate Ringer or distilled water. Since a change of 1 μ in the steady level was easily detectable, the tonicity of the cell bathed in sulphate Ringer inside and distilled water outside was at least 21/22 of that of sulphate Ringer (using the mean value of 21 μ for V_o from Table II); this puts an upper limit on the water permeability of the skin towards the outside solution, of about 1/21 of that of the inner membrane, or of about 1×10^{-4} cm/sec.

Volume changes following the substitution of chloride Ringer for sulphate Ringer

When the sulphate Ringer bathing the inside of the skin was replaced by chloride Ringer there was an initial shrinkage, of the rate and amount expected from the relative tonicities of the two solutions (cf. Table I), but this was followed by swelling as chloride entered the cell (see Fig. 3). The final cell chloride concentration could be calculated from the cell volumes and the tonicities of the respective solutions, using the equation

$$Cl_c = (V_c \cdot \pi_c - V_s \cdot \pi_s)/V_c \cdot 2$$

where V_c and V_s are the stabilized cell volumes in chloride and sulphate Ringer respectively, and π_c and π_s are the osmolarities of the corresponding solutions. The equation is derived on the assumption that 1) the cells are Cl-free after the pre-equilibration and 2) that Cl$^-$ enters the cells with an equivalent amount of K$^+$. It was found that, in general, the cell volume and chloride concentration were independent of the tonicity of the outside solution, but were
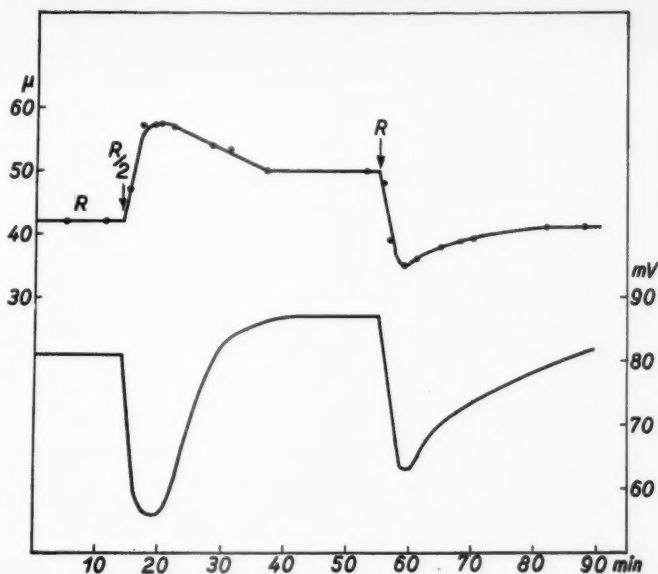
Fig. 4. Volume and potential changes on dilution of the inside medium: chloride Ringer.
   Outside medium 1/20 chloride Ringer throughout.
   Ordinate, upper curve: thickness of the epithelium in micra; lower curve: skin potential in millivolts.
   Abscissa: time in minutes. Inside medium first ordinary Ringer (R), then half Ringer (R/2) and finally Ringer again.

dependent of the outside sodium chloride concentration. This is shown in Table III, in which the mean volumes and chloride concentrations are given for the skins with chloride Ringer inside, and either high NaCl or no NaCl outside.

Such observations clearly support the contention that the outward facing membrane is permeable to Na^+ and Cl^- but not to $SO_4^=$. Fig. 3 shows the potential and volume changes associated with changes in the ionic composition of the outside and inside media. In the first 95 min of the experiment the outside medium (sulphate Ringer) is unchanged whereas the inside medium is first sulphate Ringer and then in succession half sulphate Ringer, sulphate Ringer and chloride Ringer. The slight increase in potential following the dilution of the sulphate Ringer probably is due to the increased potassium gradient cell/inside solution. The potential increase following the shift to chloride Ringer inside reflects the outward diffusion of chloride ions.

After 95 min the sulphate Ringer of the outside solution is replaced by a modified chloride Ringer in which half the Na^+ has been replaced by potas-

Table IV. Swelling after substitution of $^1/_2$-chloride Ringer for Ringer on the inside

	Initial swelling (μ)	Time to peak (min.)	Subsequent shrinkage (μ)	Time for shrinkage (min.)	Shrinkage as per cent of initial swelling
Range........	10 — 20	5 — 13	3 — 13	3 — 17	19 — 70
Mean of 11....	15 ± 1	8	7 ± 0.5	14	47 ± 4

Shrinkage on return to chloride Ringer

	Initial shrinkage (μ)	Time to trough (min.)	Subsequent swelling (μ)	Time for swelling (min.)	Swelling as per cent of initial shrinkage
Range........	7 — 19	4 — 10	3 — 8	15 — 40	19 — 50
Mean of 10....	14 ± 1	7	5 ± 0.5	28	36 ± 3

sium. The potential drops precipitously whereas the cells swell, both phenomena indicating that the membrane is readily permeable to chloride. The subsequent ionic replacements in the outside medium show that the phenomenon is reversible and depends on the simultaneous presence of Cl^- and Na^+. In some skins in very good conditions this volume change with outside chloride concentration was not found. It appeared that a few skins are capable of maintaining a low cellular chloride concentration even with full chloride Ringer outside, either by a lower sodium chloride permeability at the outer membrane, or by a more efficient cation pump. It may be that this capability is more widespread in skins which have not been exposed to sulphate Ringer for some hours.

Changes in the tonicity of the inside bathing solution: chloride Ringer

In this case the new steady state set up after a change in tonicity of the inner bathing solution is reached by movement of both water and ions, into and out of the cell respectively, and therefore the volume changes may be expected to show differences from those in sulphate Ringer, where, in the absence of a penetrating anion, only water moves. In the experiments discussed here the outside medium was 1/20 chloride Ringer throughout. A typical curve is shown in Fig. 4, and Table IV collects the characteristics of the curves obtained from 11 frogs.

From these results it is seen that not only is the maximum amount of swelling on dilution of the inside Ringer to half strength considerably less than the
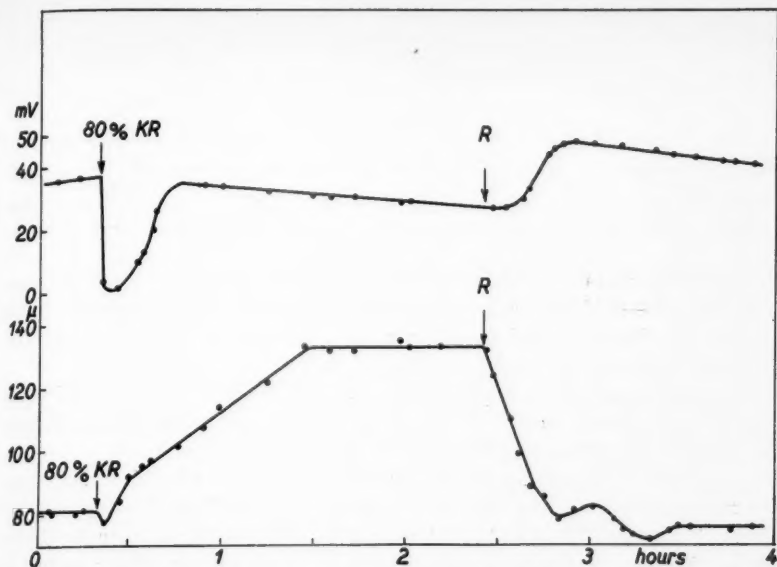
Fig. 5. Volume and potential changes produced by altering the K/Na ratio of the inside chloride Ringer solution (outside medium 1/20 Ringer).

Ordinate, upper curve: skin potential in millivolts, lower curve: thickness of the epithelium in micra.

Abscissa: time in hours.

At zero time the inside medium is ordinary Ringer. At the first arrow this medium is changed to one in which 80 per cent of the sodium had been replaced by potassium (80 per cent KR). At the second arrow the inside medium was changed back to ordinary Ringer.

normal osmotic volume in Ringer ($21 \pm 2 \mu$, Table III), but also that the curves show a biphasic course resulting in a pronounced peak, the new steady level being reached after a mean shrinkage of 7μ. The volume of the cells on return to Ringer shows very similar biphasic changes in reverse. This suggests that the ions whose loss from the cell placed in 1/2-Ringer leads to a reversal of the direction of water movement may be restored to the cell from Ringers. Since it has been shown that the inside surface of the skin is much more permeable to potassium than to sodium (KOEFOED-JOHNSEN and USSING 1958), it is reasonable to assume that KCl is the salt involved in the process. This was confirmed by measuring the K^+ concentration by flame photometry of the solution. Potassium was, indeed, lost from the skin in 1/2-Ringer to the inside solution. The skin was tied on the plastic ring with the inside facing upwards and left to equilibrate for one hour with Ringer inside and 1/20 Ringer bathing the outside of the skin. After a brief rinse on the inside of the skin with Ringer, 3 ml of 1/2-Ringer were poured on and left for 1/2 hour.

Table V. Swelling after substitution of 80 per cent K-Ringer on the inside

	Swelling (μ)		Rate of swelling (μ/h)	
	Range	Mean	Range	Mean
Rana temporaria	25 — 48	32 ± 4 (5)	36 — 72	54 ± 6 (5)
Rana esculenta	52, 53	(2)	33, 40	(2)

This solution was then withdrawn and analysed for potassium. A loss of potassium of 0.09—0.13 μmoles per cm² of skin (4 values) was calculated from the results; the loss predicted from the osmotic volumes in Ringer and in 1/2-Ringer is 0.08 ± 0.02 μmoles per cm².

The significance of the biphasic course and the peak will be more fully discussed later. It was seen in all normal dilution experiments with 1/2-Ringer but could be abolished by poisoning the skin with strophanthin.

The water permeability of the inner membrane in chloride Ringer was estimated from the initial slope of the swelling or shrinkage. The mean value from 12 skins was $(23 \pm 2) \times 10^{-4}$ cm/sec. Thus there seems to be no significant difference between the water permeabilities of the inner membrane bathed in chloride and in sulphate Ringer solutions. (See, however, the discussion.)

Effect of replacing Na^+ by K^+ in the inside solution

When the chloride Ringer bathing the inside of the skin was replaced by a mixture of normal chloride Ringer and potassium Ringer, large, regular, reversible swelling resulted if the outside solution was distilled water, or dilute Ringer solution. The curves obtained in one such experiment are shown in Fig. 5 and the results summarized in Table V.

With Ringer outside, the effect of substitution of some potassium for sodium in the inside solution was very variable; some skins showed little swelling under these conditions, some gave very irregular oscillatory swelling, and a few skins gave smooth curves, but with slower and smaller swelling than with very dilute solutions outside. This can be qualitatively explained on the basis of our assumptions, although the system has too many parameters to permit a quantitative explanation of these experiments as yet.

Volume changes associated with short-circuiting the skin

Some experiments were done in which the volume was measured during alternate periods of normal skin potential and short-circuiting. A small reproducible volume change was associated with the change in electrical conditions but both swelling and shrinking were found. Most skins swelled by 2—4 μ on short-circuiting (6 skins), but in 2 experiments a shrinkage of 3—4 μ was observed.
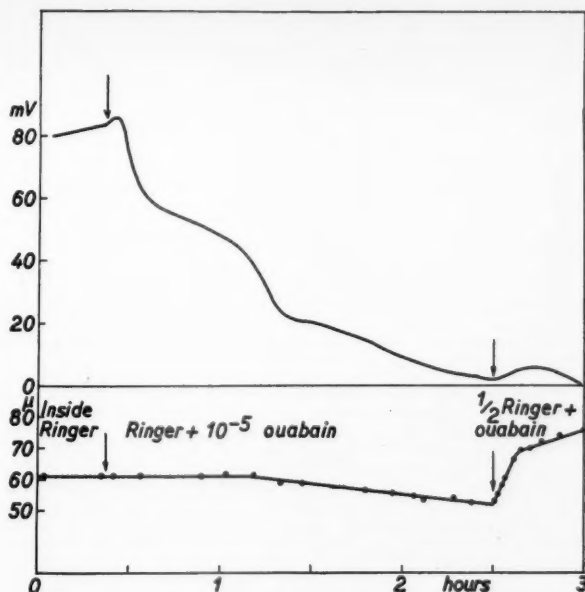
Fig. 6. Volume and potential changes produced by ouabain (g-strophanthin) 10^{-5} molar, in the inside solution.

Ordinate, upper curve: skin potential in millivolts; lower curve: thickness of epithelium in micra.

Abscissa: time in hours.

Outside medium: chloride Ringer throughout.

Inside medium: initially Ringer.

At the first arrow there is a switch to Ringer containing 10^{-5} molar ouabain. After the second arrow the medium is 1/2 Ringer with 10^{-5} molar ouabain.

Effect of ouabain in the inside solution

Ouabain, in a concentration of 10^{-5} M, has been shown to inhibit active sodium transport in the frog skin (KOEFOED-JOHNSEN 1957) and its effects on volume and potential were therefore studied. Typical volume and potential changes are shown in Fig. 6, in which ouabain is added to the inside of a skin bathed in chloride Ringer on both sides. The main potential change occurred much earlier than the volume change; after a rise of 1—2 mV over the first 3—5 min, there was a rapid decline of potential over the next 7—10 min, followed by a slow fall to zero over several hours. In 6 exp. there was no volume change for 30—50 min after adding ouabain, but in 2 exp. there was some swelling during this period, of 1 μ and 4 μ respectively. In 4 exp. in which no further solution change was made this period of steady volume was followed by shrinkage (of 6 μ, 7 μ, 8 μ and 13 μ respectively) similar to that shown in Fig. 6. Fig. 6 also shows that the characteristic biphasic volume change
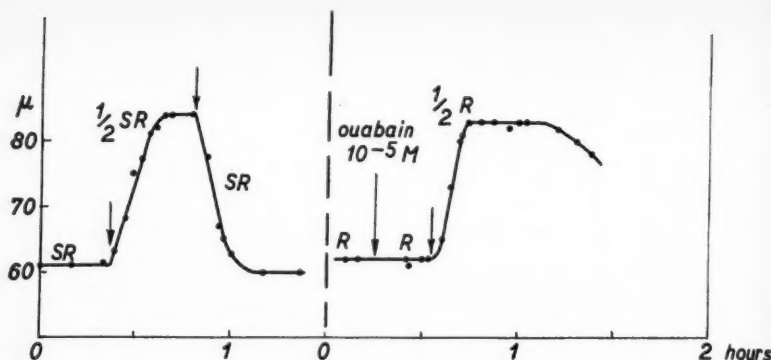
Fig. 7. Swelling of the epithelium in half chloride Ringer (inside) after ouabain treatment, compared with swelling in 1/2 sulphate Ringer.

Ordinate: thickness of epithelium in micra.

Abscissa: time in hours.

In the first experiment shown the outside bathing solution is sulphate Ringer. The inside solution is first sulphate Ringer, then after the first arrow 1/2 sulphate Ringer and then sulphate Ringer again. In the second run with the same skin the outside and inside media are both ordinary chloride Ringer. At the first arrow the inside medium is switched to Ringer containing 10^{-5} molar ouabain. At the second arrow the inside medium is changed to the same concentration of ouabain in 1/2 Ringer.

normally produced by application of 1/2-Ringer is replaced by swelling only after treatment with ouabain long enough to produce the above mentioned shrinkage.

During the first period after ouabain treatment the permeability of the inner membrane to KCl is abnormally low as seen from the fact that the cells do not swell as they do in the absence of ouabain with 80 per cent K-Ringer inside. The effect on KCl permeability during this period was also shown in 4 exp. in which the swelling in 1/2-Ringer was measured. Fig. 7 shows one such curve in which the maximum swelling in 1/2-Ringer is very close to that predicted in the absence of salt movement.

Low pH

Low pH in the inside solution has been shown to inhibit the sodium transport (SCHOFFENIELS 1956), and a concurrent effect on cell volume might therefore be expected. No such effect was found. When Ringer, buffered to pH 5.9 with 5 per cent phosphate buffer, was applied to the inside of the skin the potential fell rapidly but no swelling of the cells was observed. Since inhibition of the pump without swelling suggests that the permeability of the membrane has been greatly reduced, the water permeability was also measured as a function of pH. It was found that at pH 5.9 the permeability of the inner membrane

Table VI. Effect of pH on water permeability

pH	Water permeability of inner membrane (10^{-4} cm/sec.)
5.9	9 ± 1 (7)
8.0	17 ± 1 (10)

to water was reduced to about 50—60 per cent of its value at pH 8, and that the change was reversible. Table VI summarizes the results. (The difference between this value at pH 8 and that quoted earlier is a seasonal difference in the frogs.)

Effects of anti-diuretic hormone

The effects of anti-diuretic hormone were studied by adding insipidin[1] to the inside bathing solution (100 I. U. per 1). This substance is known to increase the osmotic permeability of the skin while leaving the diffusion permeability to water nearly unchanged, by increasing the pore size in one or more membranes within the skin (KOEFOED-JOHNSEN and USSING 1953).

After treatment of the skin with insipidin it was found that the cell volume in sulphate Ringer was no longer independent of the outside solution. Thus the water permeability of some layer between the epithelial cells and the outside solution must have been increased by hormone treatment. The water permeability of the outside membrane of hormone treated cells was obtained from the initial rate of swelling when distilled water replaced sulphate Ringer on the outside, the inside being sulphate Ringer throughout. The water permeability of the inside membrane was determined from swelling rates as described in p. 9. An independent value for the ratio of the water permeabilities of the two membranes could be obtained from the equilibrium values of the volume changes in these experiments since the degree of swelling must be determined by the condition that the osmotic flow through the outer membrane equals that through the inner membrane. This again requires that:

$$P_o \times \pi_e = P_i \times (\pi_i - \pi_e) \text{ or}$$
$$P_o/P_i = (\pi_i - \pi_e)/\pi_e$$

where P_o is the water permeability of the outer membrane, P_i that of the inner membrane, whereas π_e is the osmotic pressure in the cell and π_i the osmotic pressure of the inside solution. π_e is calculated on the assumption that the cellular contents of solutes remains constant even after hormone treatment. The values for the permeability ratio calculated according to the latter method were in good agreement with those obtained from the rates of swelling (see Table VII).

[1] Commercial preparation of vasopressin from Alfred Benzon A/S, Copenhagen.

Table VII. Water permeabilities of hormone treated skins

Exp.	Measured water permeability: inside μ/sec.	Measured water permeability: outside μ/sec.	Ratio of permeabilities (from rates of swelling)	Ratio calculated (from degree of swelling)
A	22	6.5	3.4	3.3
B	26	4.1	6.3	7
C	26	2.2	12	13
D	20	2.3	9	4

Discussion

The results of the effects of tonicity changes in sulphate Ringer indicate that of the 58 μ epidermis only 21 μ is osmotically active, the other 37 μ taking no part is osmotic changes. This might be explained if it is assumed that mainly the basal layer of cylindrical epithelium cells, the stratum germinativum, responds to osmotic changes, and that the outer cornified or cornifying cells are osmotically inert either due to low water permeability or to excessive permeability to water and electrolytes or because they are mechanically resistant to volume changes. Furthermore there must be a certain non-osmotic volume in the epithelium due to its contents of protein and other solids. Additional work is needed to clarify the relative importance of these three factors in restricting the effective osmotic volume.

The calculated thickness equivalent of the osmotic volume is in good agreement with the actual thickness of the stratum germinativum as measured in histological specimens (see for instance ENGBÆK and HOSHIKO 1957). The observed value of the water permeability of the inner membrane, 24×10^{-4} cm/sec, is about the fifth of that reported by SIDEL and SOLOMON (1957) for the human red cell, but is within the range given by PRESCOTT and ZEUTHEN (1953) for various egg cells. It is also considerably less than the value given by KAMIYA and TAZAWA (1956) for *Nitella*. It is possible, however, that the method for measuring the rate of swelling underestimates the water permeability of the inner membrane since the connective tissue of the skin is interposed as a slowly mixing layer of 0.2 to 0.5 mm thickness between the bathing solution and the epithelium. The diffusion delay in this layer for Na has a half time of 0.7 to 1.0 min (HOSHIKO and USSING 1957) which is not far from the half time for osmotic swelling of the epithelial cells.

The results of changes of the external solution show that it is the NaCl concentration of the outside solution rather than its tonicity which influences the cell volume. It could, however, be the intercellular cement round the outer cornefied cells, rather than the outer epithelial cell membranes proper, which is responsible for the (very low) water permeability of the skin to the outside.

The increase in water permeability after insipidin treatment must be produced by an increase in pore size in some layer towards the outside of the skin, even though the hormone is effective only when applied to the inside of the skin.

The biphasic form of the curves obtained by tonicity changes in the inside chloride Ringer solution has important interpretations. In 1/2-Ringer the shrinkage following the initial water entry implies that at the peak, although water is temporarily in equilibrium across the inner membrane potassium chloride is not, and the cellular product $K_c \times Cl_c$ is higher than the product $K_i \times Cl_i$ in the inside bathing solution. This must mean that initially $K_c \times Cl_c$ was higher than $K_i \times Cl_i$ in Ringer, meaning that KCl was not in equilibrium across the inner membrane but was higher in the cell than the equilibrium level. [At the peak the total salt concentration of the cell has been reduced to half its initial value in Ringer, but the permeable ions K^+ and Cl^- have been reduced to less than half their initial sum since the osmotic volume has been less than doubled. A mathematical consequence of the relations $(K_c + Cl_c)$ peak $< 1/2\ (K_c + Cl_c)$ initial and $(K_{c\ (initial)} - K_{c\ (peak)}) = (Cl_{c\ (initial)} - Cl_{c\ (peak)})$ is the product relation $(K_c \times Cl_c)_{peak} < 1/4\ (K_c \times Cl_c)_{initial}$. This then gives the inequalities $1/4\ (K_cCl_c)_{initial} > (K_cCl_c)_{peak} > (K_iCl_i)\ 1/2\text{-}_{Ringer} = 1/4\ (K_iCl_i)_{Ringer}$ or $(K_cCl_c)_{initial} > (K_iCl_i)_{initial}$].

The cellular product K_cCl_c can only be maintained higher than the external product K_iCl_i by an active transport of either K^+ or Cl^- from the inside solution into the cell, and by a passive replenishment of the other ion. Both Na^+ and Cl^- are lower in the cell than in Ringer and therefore the concentration gradients are right for a passive entry of chloride to the cell as NaCl enters through the outside membrane, and to a much lesser extent through the inside membrane if this is somewhat permeable to sodium. Therefore it is possible to achieve this non-equilibrium state by an active transport of potassium into the cell from the inside solution. The possibility that the high cellular product K_cCl_c is maintained by an active transport of chloride from the inside solution into the cell may be discarded as there is no other anion available to produce a passive entry of potassium to the cell, against its very considerable concentration gradient, through either membrane. The volume changes and the potential changes produced by diluting the inside solution showed similar time courses, but one point should be noted from Fig. 4. When water is moved into the cell the potential rises as KCl enters the cell and falls as KCl leaves the cell, but the reverse is true when the flow of water is outward; in this case the potential rises as the cellular KCl decreases and falls if the cellular KCl is increasing. This suggests that the relative permeabilities of the inner membrane to K^+ and Cl^- somehow depend on the direction of water flow. An inward flow of water increases the ratio P_K/P_{Cl} whereas under conditions of outward water flow the membrane permeability to chloride is high relative to its potassium permeability.

The swelling produced by a replacement of some Na^+ by K^+ in the inside

bathing solution is to be expected from the model, with the inside membrane permeable to potassium but only very little permeable to sodium.

The different time course of the swelling and the shrinking in Fig. 5 can be predicted on theoretical grounds. Since the cellular product $K_e \times Cl_e$ is maintained higher than the product $K_i \times Cl_i$ of the inside solution the gradient of KCl producing the volume change is not simply the change in the external product $K_i \times Cl_i$. For the swelling the effective gradient is less than the external change by an amount δ, the difference between $K_e \times Cl_e$ and $K_i \times Cl_i$ in Ringer, and for the shrinkage the effective gradient is greater than the external change by an amount δ, the difference between $K_e' \; Cl_e'$ and $K_i' \; Cl_i'$ in 80 per cent K-Ringer. On this account one would expect the shrinkage to be much faster than the swelling, as is indeed observed, but the possibility remains that the ionic permeabilities depend on the direction of the gradient.

The effect of short-circuiting must depend on the relative ion permeabilities of the two membranes, and there would seem to be enough variables to explain, qualitatively, both swelling and shrinkage as found in the experiments. At the moment a quantitative description of these changes does not seem possible.

Stoppage of the pump, unaccompanied by any permeability changes can only lead to swelling of the cells, since NaCl continues to leak into the cell from the outside and the initially balanced diffusion of KCl to the inside solution can only decrease with time. Therefore the lack of volume changes during the first hour or so of ouabain treatment can only be explained on the basis of permeability changes as well as stoppage of the pump, and suggest that both membranes become relatively tight to ions during this period. The tightness of the inner membrane is shown by the experiments with 80 per cent K-Ringer and 1/2-Ringer in this period. The fact that the ouabain treated skin responds to 80 per cent K-Ringer on the inside by a potential drop (like normal skin) shows that at the inner membrane $P_K > P_{Cl}$, and therefore the rate of KCl movement will be limited by P_{Cl}, the lower of the two ion permeabilities. Hence the reduced permeability to KCl implies a reduced chloride permeability of the inner membrane, an effect confirmed by the fact that the behaviour of the inner membrane is closer to that of an ideal potassium electrode after ouabain than before. During the second period in ouabain the KCl permeability of the inner membrane appears to be restored or increased, but to account for the shrinkage it appears that the outer membrane is still tight to NaCl.

Similar permeability changes seem to be associated with inhibition of the pump by low pH in the inside solution, without any concurrent swelling. This, together with the results of the water permeability of the inside membrane at low pH, suggests that the tightening is a general one to both water and ions. It may be that any interference with the pump mechanism is accompanied by a decrease in the passive permeabilities to ions and that the active ion transport and the passive fluxes are not entirely independent.

Although in most cases a quantitative explanation of the results cannot be produced from so many variables, it does seem from these experiments that measurements of volume changes in the epithelial cells, in conjunction with electrical measurements, does provide useful information on which to base any interpretation of the processes concerned.

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A Comparison of the Efficiencies of Free Lysine and of Roller-Dried Skim Milk, Fish Protein and Soya Bean Protein for the Supplementation of Wheat Bread

By

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Abstract

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ciencies of free lysine and of roller-dried skim milk, fish protein and soya bean
protein for the supplementation of wheat bread.* Acta physiol. scand. 1961.
53. 366—375. — Free L-lysine · HCl, roller-dried skim milk, fish
protein and soya bean protein were added to the dough of wheat
bread and their supplementary values for the improvement of the
protein value of the bread were compared. The growth rates and
nitrogen efficiency ratios of young albino rats were used as indices
of the improvements obtained.

Within the concentration range studied, the supplementary values
of the added substances were directly proportional to the amount
of available lysine which they provided. No beneficial effect from
the other essential amino acids supplied by the proteins tested as
supplements was detected. The data also indicate that roller-dried
skim milk suffered a considerably greater loss of lysine during the
baking of wheat bread than did the other supplements.

The principle of amino acid supplementation of low quality protein is to
add such an amount of the most limiting amino acid that it increases the
total content of this acid in the diet to a level where — from a nutritional
point of view — it balances the second most limiting amino acid. Due to the

366

"law of diminishing returns", which in this case may be understood and interpreted in terms of the laws of enzyme kinetics, it is uneconomical from a practical point of view to add exactly this amount; a slightly smaller quantity will give an optimum result. No beneficial effect should in such a situation result from the addition of the second most limiting amino acid or from other added amino acids. One would therefore, at least as a first approximation, assume it to be unimportant whether or not the most limiting amino acid was supplied free or protein-bound.

In connection with experiments on the amino acid supplementation of wheat bread, we have also studied the supplementary value of a few proteins, *viz.* milk, fish and soya bean proteins. The results of comparisons of amino acid and protein supplementations will be presented in this paper and discussed in the light of the principles mentioned above. Other factors that might influence the outcome and interpretation of such comparisons will also be discussed.

The growth rates and nitrogen efficiency ratios[1] of young albino rats which were given the various wheat bread diets were used as indices of the effects due to the supplements. Both the amino acids, principally lysine, and the proteins were incorporated into the dough of the bread. This was considered to be a more realistic approach than simply to add the supplements to the diets since in practice, most products to be supplemented would be heat treated in some way, *e. g.* cooked, baked, fried, toasted etc., before being consumed.

Experimental

Analytical methods. The lysine contents of the various dietary ingredients and the diets were determined microbiologically using *Leuconocstoc mesenteroides* P-60 (ATCC 8042) as the test organism. The Bacto Lysine Assay Medium from the Difco Laboratories, originally developed by STEELE *et al.* (1949) was employed. However, the incubation temperature of the tubes was 34° C (SCHIAFFINO, McGURIE and LOY 1958) instead of 37° C as suggested by STEELE *et al.* (1949). The content of threonine was also determined in some cases using *Streptococcus faecalis* (ATCC 8043) and the Bacto Threonine Assay Medium (STEELE *et al.* 1949). The details of the procedures used, including the conditions for the extraction and hydrolysis of the samples, are described in a previous paper (ERICSON, LARSSON and LID 1961).

Nitrogen was determined by the Kjeldahl method as modified by PERRIN (1953). The ammonia was collected in boric acid and titrated with 0.01 N HCl.

Dry weight determinations were carried out by heating the samples at 104—105° C for 24 h and cooling them in a desiccator over silica gel.

Bread formulae and baking conditions. Two different series of rat experiments will be described in the following. The basal formula of the bread for the first series was:

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[1] *Nitrogen* efficiency ratios (*i. e.* gain of weight per g of nitrogen consumed) were preferred instead of *protein* efficiency ratios partly because the exact correlation between nitrogen and protein for the different ingredients of the diets, such as wheat flour, milk, fish and soya protein, was not known and partly because the added free amino acids increased the nitrogen but not the protein content of the diets.

```
water.................................    1,000 g
wheat flour¹ (70 % extraction)........    1,900 g
lard..................................       20 g
roller-dried skim milk (< 1 % fat)........    50 g
sugar.................................       10 g
salt..................................       20 g
malt extract..........................       10 g
yeast ................................      200 g
```

For the second series, which was performed more than a year later, the formula was slightly modified partly to suit the quality of the flour then available:

```
water.................................    1,000 g
wheat flour¹ (70 % extraction).........    1,850 g
lard..................................       20 g
roller-dried skim milk (< 1 % fat).....      50 g
sugar.................................       20 g
salt..................................       20 g
yeast ................................      100 g
```

These formulae were modified by the addition of amino acids or proteins as will be described later. The amino acids — L-lysine · HCl (Pfizer & Co. Inc.) and DL-threonine (Fluka, A. G.) — were dissolved in part of the water for the dough. The proteins — roller-dried skim milk (from the Swedish Milk Products, Stockholm, Sweden), fish protein ("Sunco-protein", from AB Sunco,[2] Mölndal, Sweden) and soya bean protein ("Promine D" from Central Soya Co., Inc., Chicago, USA) — were added to part of the flour before being mixed into the dough. The dry weights, nitrogen and lysine contents for the three proteins tested were determined and found to be 96.8 %, 95.0 % and 92.5 %; 5.60 %, 13.8 % and 13.8 %; and 2.86 %, 7.48 % and 5.92 %.

All bread was pan baked. Dividing, rounding and moulding were done by hand. Proofing took place at 45° C for about 30—50 min and at a relative humidity of approximately 85 %. The baking temperature was 190—220° C and the baking time 20—30 min. Each loaf of bread weighed about 400 g.

After baking, the bread was sliced and dried. In the first series of experiments this took place at 25—27° C for 2 days. The breads used in the second series were dried at 25—40° C for 2 days. The air-dried bread was finally ground in an Electrolux "Assistent" bread mill. The dry weight of the ground bread was approximately 92 %.

The diets. The diets used in both series of experiments were composed of 91 % of the dried and ground bread, 3 % of a salt mixture (HEGSTED et al. 1941), 5 % soya bean oil, 0.5 % cod liver oil and a vitamin mixture (HARPER et al. 1953). The vitamin mixture provided in mg per 100 g of ration: thiamin · HCl 0.5, riboflavin 0.5, niacin 1.0, calcium pantothenate 2.0, pyridoxine 0.25, biotin 0.01, pteroylglutamic acid 0.02, cyanocobalamin 0.002, inositol 10, menadione 0.5 and choline chloride 150. Before being mixed into the rest of the diet, the vitamins were distributed in the salt mixture and a small quantity of finally powdered bread. The cod liver oil supplied approximately 375 I. U. vitamin A and 37.5 I. U. vitamin D per 100 g of diet.

Conditions of the rat experiments. In the first series of experiments, the animals were housed individually in cylindrical glass vessels having a diameter of 20 cm and containing wood shavings. The temperature in the animal room was 27° C. The rats were males of the Wistar strain with an average initial weight of 76 g.

¹ Alexandra, Kungsörnen AB, Stockholm, Sweden.
² Daughter Company of AB Astra, Södertälje, Sweden.

Table I. Composition of diets and growth rates for the groups of rats in Series I. Twelve rats per group were used initially. The additions of roller-dried skim milk, L-lysine·HCl and DL-threonine were made before baking

Group	Diet Additions expressed in per cent of the fresh weight of the flour	Determined content of			Growth rate g/day
		Nitrogen[1] %	L-lysine ·HCl[1] %	L-threonine[1] %	
A	Basal (which contains 2.63 % roller-dried skim milk)[2]	2.27	0.51	0.39	[3]1.87 ± 0.06
B	As A, but with a total of 10.5 % roller-dried skim milk.	2.51	0.76	0.48	2.70 ± 0.08
C	As A, but with a total of 21.0 % roller-dried skim milk.	2.79	1.00	0.55	3.04 ± 0.04
D	As A, but with a total of 26.3 % roller-dried skim milk.	2.87	1.03	0.57	3.33 ± 0.04
E	As A, but with 0.55 % L-lysine·HCl	2.27	0.90	0.37	3.13 ± 0.03
F	As A, but with 0.55 % L-lysine·HCl and 0.22 % DL-threonine	2.31	0.93	[4]0.44	3.86 ± 0.03

[1] In per cent of the dry weight of the diet.

[2] This amount of roller-dried skim milk corresponds to the 50 g/l water in the dough which was always used. For the groups B, C and D, the amount of dry milk corresponds to 200, 400 and 500 g/l.

[3] Standard error of the mean.

[4] The loss of free L-threonine during the hydrolysis preceding the microbiological determination has been found to be of the order of 25—30 % (ERICSON, LARSSON and LID 1961).

The second series was carried out in a different animal room having wire bottom cages, a temperature of 25° C and a relative humidity of approximately 50 %. The rats for this experiment were Sprague-Dawley males and had a weight of 60 g at the time when they were given the experimental diets.

In both series the diets were fed *ad libitum*. The rats were given a non-supplemented bread diet for a few days before receiving the experimental diets. This was done mainly in order to accustom the rats to bread diets.

Results

Due to difficulties in obtaining rats suitable for growth rate experiments, the first series of experiments reported here had to be performed with rats having a higher initial weight than those used in the second series (see above). This influences the absolute values of the growth rates obtained. Since this should not, however, have any influence on the problem under study — *viz.* a comparison of the supplementary values of protein and free amino acids — the results of this series are presented although a direct comparison with the data of the second series of experiments is not possible.

Table II. Composition of diets, growth rates and nitrogen efficiency ratios for the groups of rats in Series II. Ten rats per group were fed the different diets for 21 days. All additions were made before baking

Group	Diet Additions listed below are expressed in per cent of the weight of the flour	Determined content of		Average gain in weight g	Average gain in weight per g nitrogen consumed g/g
		Nitro-gen[1] %	L-lysine ·HCl[1] %		
A	Basal (which contained 2.70 % roller-dried skim milk)	2.21	0.55	36.7	[2]8.4 ± 0.1
B	Basal + 0.22 % L-lysine·HCl.	2.26	0.80	71.2	12.8 ± 0.3
C	Basal + 0.44 % L-lysine·HCl.	2.29	0.95	102.3	15.3 ± 0.3
D	Basal + 5.80 % roller-dried skim milk. . . .	2.39	0.74	54.2	9.9 ± 0.2
E	Basal + 11.6 % roller-dried skim milk. . . .	2.59	0.81	69.6	10.8 ± 0.3
F	Basal + 1.95 % fish protein	2.44	0.70	66.1	11.1 ± 0.2
G	Basal + 3.90 % fish protein	2.62	0.82	97.5	13.2 ± 0.2
H	Basal + 6.00 % soya bean protein.	2.84	0.79	105.2	12.6 ± 0.2

[1] In per cent of the dry weight of the diet.
[2] Standard error of the mean.

Six groups of rats, originally comprising 12 animals each, were used in the first series of experiments. The first group was given the basal bread diet (which contains 50 g roller-dried skim milk per 1 of water in the dough), the second, third and fourth groups diets with bread containing a total of 200, 400 and 500 g respectively of roller-dried skim milk per 1 of water in the dough, the fifth group a diet with a bread containing 0.55 % L-lysine · HCl of the flour weight, and the sixth group a diet with a bread containing 0.55 % L-lysine · HCl and 0.22 % DL-threonine. The amounts of roller-dried skim milk used in the second, third and fourth groups correspond to a total of 10.5, 21.0 and 26.3 % of the fresh weight of the flour. The contents in the diets of nitrogen, L-lysine · HCl and L-threonine were determined and are shown in Table I.

Six rats per group were killed after 21 days and the remainder after 49 days. Determinations of liver fat and histological examinations of the livers were carried out in all cases. The results of these observations together with those from other rat experiments will be presented in a forthcoming paper (LARSSON, RUBARTH and ERICSON 1962).

The average growth rates of the various groups are given in Table I. It can be seen that a progressively increasing rate of growth resulted from the addition of increasing amounts of roller-dried skim milk to the dough. The
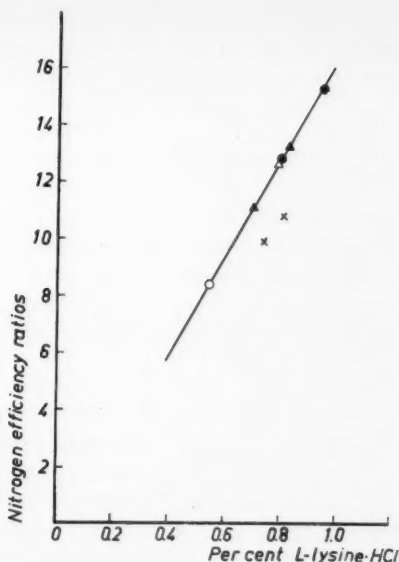
Fig. 1. Nitrogen efficiency ratios for the groups of rats in Series II as a function of the content of L-lysine·HCl in the diets.
○ Group A, ● group B, ● group C, × group D, × group E, ▲ group F, ▲ group G and △ group H.

effect of the addition of L-lysine·HCl alone (group E) was slightly larger than the effect of the addition of 400 g roller-dried skim milk (group C) although the latter group supplied a greater amount of lysine (expressed as L-lysine·HCl). The roller-dried skim milk provided in addition a significant quantity of threonine — the second most limiting amino acid in wheat bread (ERICSON 1960) — but it is obvious that this was without effect. This observation indicated that there was a considerable loss of the lysine in the roller-dried skim milk during the baking, an inference that is supported by a comparison of the groups D and F. Group D, in which the added lysine and threonine came from roller-dried skim milk, supplied more of both these two amino acids than group F. Despite this, group F was clearly superior to group D.

In the second series of rat experiments, 8 groups of rats each comprising 10 animals were used. The first group received a basal bread diet and the subsequent seven groups were provided with diets made from breads supplemented with two levels of free L-lysine·HCl, two levels of roller-dried skim milk, two levels of fish protein and one level of soya bean protein as shown in Table II. This table also gives the nitrogen and lysine (expressed as L-lysine··HCl) contents of the diets, the average gain of weight and nitrogen efficiency ratios of the rats in the different groups. The rats were given the various experimental diets for 21 days.

What stands out primarily from an examination of the data in Table II is that, in comparison with the other supplements, the addition of roller-dried

skim milk failed to promote growth and nitrogen efficiency ratios in proportion to the lysine content of the corresponding diets. For the other supplements, a good correlation between the lysine content of the diet, as determined microbiologically, and the nitrogen efficiency ratio is indicated. This becomes more obvious when the nitrogen efficiency ratios of the different groups are plotted as a function of the lysine content of the corresponding diets (Fig. 1). For all groups, with the exception of the two groups of rats that received diets containing additional roller-dried skim milk, the nitrogen efficiency ratios were directly proportional to the total lysine content of the diets regardless of whether the lysine had been supplied free or protein-bound. The groups given the diets fortified with dry milk had nitrogen efficiency ratios that were less than expected from the lysine content of the diets.

Discussion

The data obtained in the present investigation demonstrate that the improvement of the protein value of wheat bread resulting from the addition of various supplements is determined by the content of available lysine in the final product, as long as lysine remains the most limiting amino acid. The relationship appears to hold independently of whether the supplementary lysine is provided by lysine-rich proteins or in the form of L-lysine · HCl. The same conclusion has been reached and explicitly stated by HOWARD et al. (1958), BROWN et al. (1959) and GUGGENHEIM and FRIEDMANN (1960) and is apparent also from the work of LIGHT and FREY (1943), HUNDLEY, ING and KRAUSS (1956), SABISTON and KENNEDY (1957), HUTCHINSON, MORAN and PACE (1959) and SCHWARTZ, TAYLOR and FISHER (1959). DESHPANDE, HARPER and ELVEHJEM (1957), on the other hand, state that "the finding that a mixture of essential amino acids simulating fibrin was not able to support as good a growth as the intact protein indicates some superiority of intact protein over amino acids as a dietary supplement (to wheat flour) under the conditions of the present experiments".

The assumption that free lysine and protein-bound lysine are nutritionally equivalent and that no effect results from amino acids other than the most limiting supplied by the protein is implicit in the numerous attempts that have been made to determine the lysine availability in various foods (cf. SCHWEIGERT and GUTHNECK 1953, CARPENTER et al. 1957, KRATZER and GREEN 1957, TSIEN, JOHNSON and LIENER 1957, MAURON and MOTTU 1958, GUPTA et al. 1958, OUSTERHAUT, GRAU and LUNDHOLM 1959, CALHOUN, HEPBURN and BRADLEY 1960, MUELENAERE and FELDMAN 1960 and HELLER et al. 1961). One factor that could make such determinations uncertain would be a substantially different rate of absorption of the free and the protein-bound lysine. Only limited data seem to be at hand on this point but LONGENECKER and HAUSE (1958) and SCHWARTZ, TAYLOR and FISHER (1959) have

studied the problem in dogs and chicks and report that free and bound lysine are absorbed at a similar rate. Another factor influencing comparisons of the apparent nutritive values of free and protein-bound amino acids is the circumstance that the requirement of an amino acid becomes greater when the protein (or amino acid) content of the diet is increased (cf. GRAU 1948, ALMQUIST 1949, KRATZER, WILLIAMS and MARSHALL 1950, BIRD 1953, SALMON 1954, BRESSANI and MERTZ 1958, MUNAVER and HARPER 1959, McWARD et al. 1959, SWENDSEID, HARRIS and TUTTLE 1960). In the experiments reported here, this phenomenon has apparently not greatly affected the results, probably due to the rather small increase in the protein content of the diets resulting from the protein supplements.

The correlation between protein value and lysine content discussed above and shown in Fig. 1 would of course be valid only up to the level of lysine which satisfies the requirement of the animal under study. Above this level one would expect to obtain a beneficial effect also from the second most limiting amino acid (in the case of wheat, threonine) furnished by the supplementary protein. A comprehensive study of the supplementation of rice with its two most limiting amino acids, i. e. lysine and threonine, has been performed by ROSENBERG, CULIK and ECKERT (1959) who presented interesting observations pertinent to this problem.

It should also be pointed out that a relationship like the one presented in Fig. 1 would hold only if the content of lysine found upon microbiological determination or a constant fraction thereof were available to the rat. This seems to be true in the cases where L-lysine · HCl, fish protein or soya protein were used as supplements but not when the bread was fortified with roller-dried skim milk. Most probably, the greater loss of lysine from roller-dried skim milk as indicated by the results of both Tables I and II is partly due to the high amount of sugar, mainly lactose, in the milk. Since HENRICSON et al. (1961) have found only small differencies in the protein values of spray-dried and roller-dried skim milk from the Swedish Milk Products, Stockholm, it seems likely that the loss of lysine occurred during baking and not during the manufacture of the roller-dried skim milk.

From a practical point of view, the choice between protein and amino acid supplementation depends on a number of factors. The content of the desired amino acid in the supplementary protein and the degree of inactivation of the protein-bound amino acid compared with the free amino acid during the preparation (e. g. baking, cooking etc.) of the supplemented product as discussed here and in a previous paper (ERICSON et al. 1961) are of obvious importance for deciding the cost of the improvement. However, other aspects such as the stability of the supplement during storage and the possibility of making the additions reproducibly and cheaply on a large scale and without impairing the taste, odour, colour and texture of the final product should be mentioned. The possible risk of introducing "imbalances" by the additions

should also be considered although this will most likely gradually become a less serious problem as more knowledge accumulates.

In earlier work (ERICSON 1960) we found that the addition of 0.4 % of L-lysine · HCl to a bread diet after baking improved the nitrogen efficiency ratio by 85 %. In the present study it was found that the same amount of L-lysine · HCl added before baking resulted in an improvement of 82 %.

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Studies of the Effect of X-Rays
on the Glucuronide Synthesis and β-Glucuronidase Activity
in the Duodenal Mucous Membrane of the Rat

By

K. Hartiala, V. Näntö and U. K. Rinne

Abstract

Hartiala, K., V. Näntö and U. K. Rinne. *Studies of the effect of X-rays on the glucuronide synthesis and β-glucuronidase activity in the duodenal mucous membrane of the rat.* Acta physiol. scand. 1961. *53.* 376—380. — In a series of previous experiments we have studied the effect of local X-ray irradiation on the glucuronide conjugation capacity and on the β-glucuronidase activity of the liver and gastric mucosa. According to these studies the sensitivity of these tissues towards irradiation is different. These studies also show that the changes in the glucuronide formation capacity and the β-glucuronidase activity are not parallel which is taken as a further indication that the β-glucuronidase enzyme is not involved in the synthesis of glucuronide-conjugates. In the present work we have studied the glucuronide conjugation and β-glucuronidase activity of rat duodenal mucosa after local X-ray irradiation.

Materials and Methods

One hundred thirty-two male rats (Wistar, weight range 180—220 g) were used in the experiments with 77 control animals. The local X-ray irradiation was performed in Nembutal anesthesia, the duodenum was exposed through a mid-line excision, and the animal protected with a 3 mm lead shield. The local X-ray irradiation was performed by single 400 r (group A) and 1,200 r (group B) doses (185 kV X-ray machine, 10 mA, 0.5 cm Cu), as described previously (Hartiala, Näntö and Rinne 1958, 1959 and Hartiala *et al.* 1960).

Prior to and after the operation and irradiation the animals were fed with usual laboratory diet and water ad libitum. All of the animals survived for the later analyses.
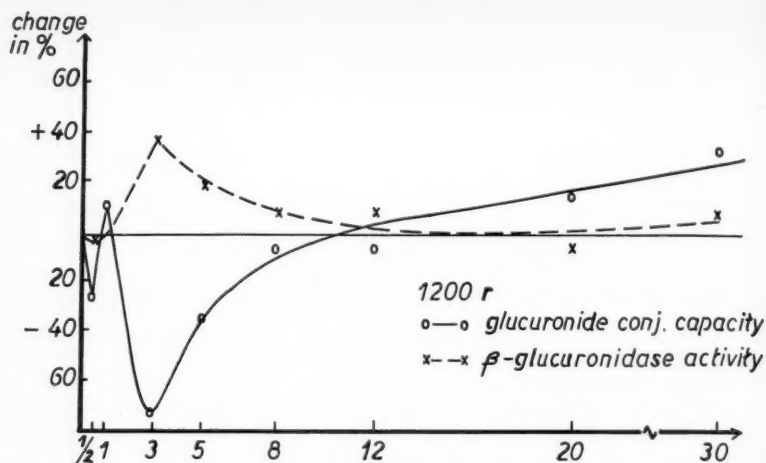
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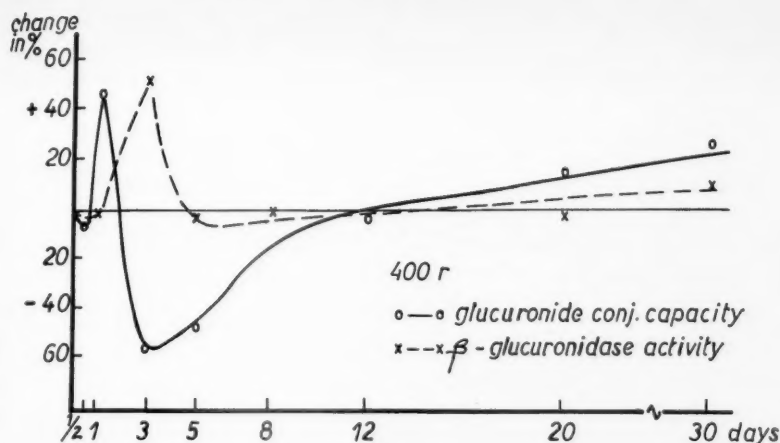
Fig. 1. Changes in duodenal glucuronide conjugation capacity and β-glucuronidase activity expressed as per cents at various times after local X-ray irradiation.

The animals were killed in 8 groups, 12 hours, and 1, 3, 5, 8, 12, 20 and 30 days after the irradiation. Specimens were taken from the mucous membrane of the duodenum and the glucuronide synthesis measurements (according to Levvy and Storey 1949, Hartiala and Rontu 1955) and β-glucuronidase assays (Talalay, Fishman and Huggins 1946, modified by Fishman, Springer and Brunetti 1948) were performed from each animal. Duplicate determinations were made from each sample. In the statistical treatment of the results "Student's" t-test was used.

Table I. Effect of local X-ray irradiation on the glucuronide synthesis. The results expressed as μg of o-aminophenolglucuronide produced by the duodenal mucous membrane per 100 mg dry weight tissue

Time after irradiation	Group A (400 r)			Group B (1,200 r)		
	Number of animals	μg/100 mg	P^2	Number of animals	μg/100 mg	P^2
12 hours......	12	[1]119 ± 11.1	> 0.05	6	94 ± 4.8	> 0.05
1 day	12	189 ± 18.0	< 0.01	6	145 ± 10.2	> 0.05
3 days	6	54 ± 3.5	< 0.01	6	37 ± 3.8	< 0.001
5 days	12	67 ± 3.6	< 0.01	12	85 ± 13.6	> 0.05
8 days	6	110 ± 7.6	> 0.05	12	120 ± 21.1	> 0.05
12 days	12	123 ± 14.4	> 0.05	12	119 ± 13.0	> 0.05
20 days	6	146 ± 15.6	> 0.05	6	150 ± 15.0	> 0.05
30 days	6	165 ± 18.8	> 0.05	6	175 ± 18.9	> 0.05
Control animals	77	130 ± 6.4				

[1] Standard error.

[2] P values refer to comparison with the control animals.

Fig. 2. Changes in duodenal glucuronide conjugation capacity and β-glucuronidase activity expressed as per cents at various times after local X-ray irradiation.

Results

Glucuronide formation (Table I, Fig. 1 and 2). Both after the 400 r and 1,200 r irradiation a slight decrease in the conjugation capacity was present 12 hours after the treatment. This was followed by an increase within the next 12 hours, (400 r: P < 0.01). After this the conjugation capacity diminishes greatly so

Table II. *Effect of local X-ray irradiation on the β-glucuronidase activity in the duodenal mucous membrane. The results expressed as β-glucuronidase units per g wet weight tissue*

Time after irradiation	Group A (400 r)			Group B (1,200 r)		
	Number of animals	β-glucuronidase units/g	P[2]	Number of animals	β-glucuronidase units/g	P[2]
12 hours......	12	[1]1,341 ± 92	> 0.05	6	1,320 ± 208	> 0.05
1 day	12	1,364 ± 96	> 0.05	6	1,389 ± 166	> 0.05
3 days	6	2,093 ± 235	< 0.001	6	1,911 ± 282	< 0.01
5 days	12	1,313 ± 147	> 0.05	6	1,680 ± 167	> 0.05
8 days	6	1,381 ± 115	> 0.05	12	1,498 ± 59	> 0.05
12 days	12	1,411 ± 66	> 0.05	12	1,529 ± 167	> 0.05
20 days	6	1,308 ± 195	> 0.05	6	1,297 ± 104	> 0.05
30 days	6	1,524 ± 63	> 0.05	6	1,502 ± 94	> 0.05
Control animals	77	1,394 ± 45				

[1] Standard error.
[2] P values refer to comparison with the control animals.

that the decrease is 58 per cent with the 400 r dose (P < 0.01) and 71 per cent with the 1,200 r dose (P < 0.001). The conjugation capacity approaches the control level within 8 days and 20 and 30 days after the irradiation it was above the control level. The difference, however, is not significant.

β-glucuronidase (Table II, Fig. 1 and 2). After a slight initial decrease 12 hours after the treatment the enzyme activity rises being at its maximum 3 days after the irradiation. The increase is 50 per cent with the 400 r dose (P < < 0.001) and 37 per cent with the 1,200 r dose (P < 0.01). After this the activity settles down to the control level this being more rapid after the smaller irradiation dose. On the later periods no differences with the control values were noted.

Discussion

The duodenum is the most radio-sensitive part of the intestinal tract (DES-JARDINS 1932, PIERCE 1948, LESHER 1957). In rats exposed to 600 r of total-body X-irradiation, degenerative changes and cell death in the duodenal epithelium are seen 3 to 8 hours after the irradiation, and bizarre cells from 1 to 3 days. Recovery is very rapid: at 5 days very little residual effect is seen. The greatest damage is located to the basal cells of the crypts of Lieberkühn. The epithelium of the Brunner's glands is more resistant than the villous or crypt epithelium (PIERCE 1948).

Not much information is available of the radiation effects on the enzyme systems of the intestinal mucous membrane. FRENCH and WALL (1957) showed that rat intestinal cholinesterase activity was reduced 48 hours after irradiation. Absorption by the small intestine after irradiation is reported to be decreased (MEAD, DECKER and BENNET 1951, BUCHWALD 1931). DICKSON (1955) observed no impairment of hexokinase activity after irradiation. MOSS (1957) estimated the total phosphorylation activity of the exteriozed X-irradiated small intestine, and found a decrease in phosphorylation. KAY and ENTENMAN (1959) measured the glucose oxidation rate of rat small intestine mucous membrane, and found an increase on the 3rd and 4th post-irradiation days. They concluded that this increase after X-irradiation is due entirely to increased citric acid cycle activity.

As in our previous studies local X-ray irradiation applied to the duodenal mucosa produces severe changes in the conjugation functions. After the initial stimulation of the glucuronide conjugation the following depression reaches its maximum on the third day and is about the same magnitude as observed in similar studies with the liver. The recovery takes about one week and is actually over-compensated as in the liver. The duodenal mucosa appears to be somewhat more sensitive towards the smaller radiation dosage as the liver.

As compared to the gastric mucosa the response to radiation appears to follow a different pattern. The most obvious difference is the slower recovery in the gastric mucosa.

As to the *β*-glucuronidase activity under the same conditions the changes

do not coincide with the changes in the glucuronide conjugation capacity. In this respect the results agree with our previous observations. On the other hand such great increase in the activity as was seen in the liver as a late effect was not present.

It would thus appear that the sensitivity of the studied tissues towards irradiation is somewhat different. The different life span of the cellular elements in the various organs might explain these changes. On the other hand these studies do not reveal which part of the complex enzyme machinery in the glucuronide synthesis is involved in the observed changes. These studies are now under progress in this laboratory.

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Stimulation of Sweating by Exercise after Heat Induced "Fatigue" of the Sweating Mechanism

By

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Received 3 July 1961

Abstract

Ahlman, K. and M. J. Karvonen. *Stimulation of sweating by exercise after heat induced "fatigue" of the sweating mechanism.* Acta physiol. scand. 1961. *53.* 381—386. — When wrestlers lose weight before a competition, they often induce sweating in a hot bath until the "fatigue" of the sweating mechanism. It is reported that at this stage exercise may still induce sweating. This claim was tested in experiments on five young males. After three or four exposures of 12 min each to ambient heat in a "sauna" bath which caused the rectal temperature to rise to values in a range from 38.4 to 39.4° C, substantial sweating could no more be elicited by repeating the thermal stimulus. However, treadmill running for 20 min caused a further weight loss of 0.1 to 0.9 kg, although the rectal temperature now remained on an average 0.7° C lower than during the preceding exposure to heat. The mean total loss of weight during the whole procedure was 3.6 per cent of the initial body weight.

The concentration of sodium, potassium and chloride varied individually at repeated exposures to heat. In most subjects, the range of the variation was small. During exercise the mean content of sodium was lower than in sweat collected during the first exposure to ambient heat.

The results are discussed.

When wrestlers have to lose weight before a competition, they induce sweating in a hot bath or through cross-country running. A prolonged exposure to heat causes an apparent fatigue of the sweating mechanism. It is known among wrestlers that at this stage, further sweating may still be induced by exercise, *e. g.* by cross-country running (Ahlman and Karvonen 1961). This phenomenon does not appear to be previously mentioned in the literature on sweating.

An experimental study was made, imitating the methods of losing weight as used by wrestlers. Attention was paid to weight loss, rectal temperature and the mineral composition of sweat.
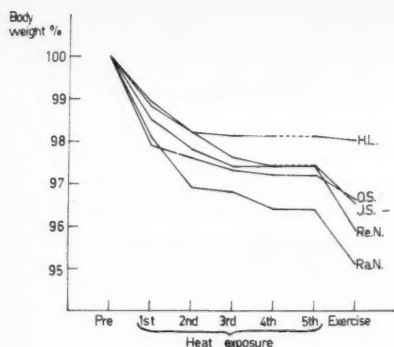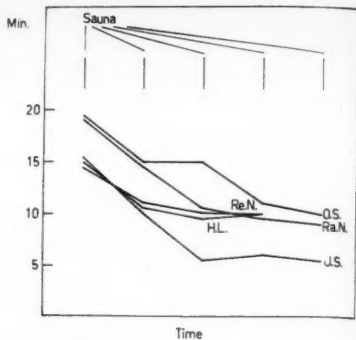
Fig. 1   Fig. 2

Fig. 1. The loss of body weight at repeated exposures to ambient heat in the sauna. After heat induced sweating had stopped, the subjects ran for 20 min on a treadmill.

Fig. 2. The duration of visible sweating after repeated exposures to heat.

Method

Five male students of physical education served as subjects. The experiment was started by weighing the subject, after he had emptied the bladder and defecated. The entire skin was thoroughly washed and dried. The right arm was enclosed in a plastic sleeve, and the rectal temperature was recorded. The subject was then exposed to intense heat in a Finnish "sauna" bath for 12 min. The rectal temperature was recorded after 9 min in the sauna.

On leaving "sauna", the volume of sweat in the plastic sleeve was measured, and a sample was taken for mineral analysis. The subject was wrapped in blankets, in order to retard the fall of body temperature. He remained supine at ordinary room temperature as long as visible sweating continued. When it had ended, the skin was again washed, dried, and the subject was weighed.

The above procedure was repeated three or four times, *i. e.* as long as significant sweating could be elicited.

After the last exposure to ambient heat, the subject ran on a treadmill for 20 min, during which sweat was again collected. At the termination of running, the rectal temperature and body weight were again recorded.

The chloride concentration in sweat was determined by using the method of SCHALES and SCHALES (1941) as modified by BRUN (1949), and the sodium and potassium with a flame photometer, using lithium as an internal standard.

Results

The total loss of weight varied from 1.5 to 3.0 kg. The amount of weight lost at each stage is shown in Fig. 1. Most weight was lost during the first exposure to heat. The mean losses at each exposure were 1.6, 0.7, 0.3, 0.1 and 0.0 per cent of the initial body weight. The "fatigue" of the sweating mechanism was seen also in a shortening of the duration of visible sweating after each exposure (Fig. 2).
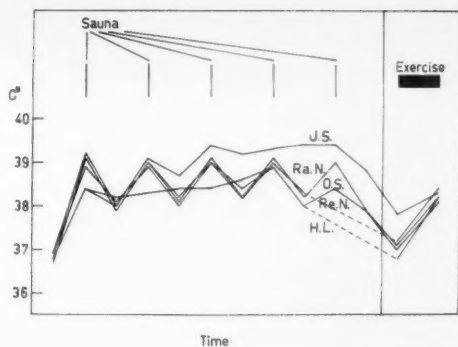
Fig. 3. Rectal temperature in sauna, during subsequent rest periods, and before and after treadmill running.
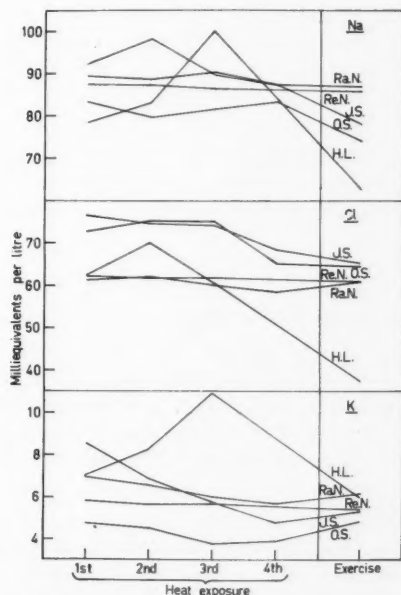
Fig. 4. Sweat potassium, sodium and chloride content during repeated exposures to ambient heat and subsequent exercise.

The rectal temperature offered no explanation to the diminishing returns of sweat; it remained essentially constant at each exposure, or showed a tendency to a rise (Fig. 3). This applied both to temperature in sauna and during the subsequent sweating at room temperature.

In all subjects, running again induced sweating. The loss of weight varied from 0.1 to 0.9 kg. The mean loss was 0.6 per cent of the initial body weight. This occurred while the rectal temperature remained essentially lower than during the exposure to ambient heat.

Table I. Sweat sodium, potassium and chloride during first exposure to heat and during exercise

Electrolyte	Milliequivalents per litre		t	P
	Heat	Exercise		
Sodium	86.0	76.8	3.05	0.05
Potassium	6.62	5.53	1.91	..
Chloride	67.0	57.8	2.05	..

The variations of the electrolyte content of sweat are shown in Fig. 4. Considerable individual variations are seen. They are, however, less marked for chloride than for sodium and potassium. In one subject, H. L., the sodium and potassium show a steep rise. In this subject, sweating stopped early and the total weight loss was smallest, 1.5 kg only.

In sweat collected during exercise, the mean content of each electrolyte was lower than in that secreted during the first stay in the sauna. However, the difference was significant only for sodium ($P < 0.05$; Table I). When the electrolyte content of the work sweat is compared with that in the immediately preceding collection in sauna, sodium again shows a consequent fall, whereas potassium and chloride show no regular tendency.

Discussion

A "fatigue" of the sweating mechanism may be produced either by prolonged and copious thermal sweating (HANCOCK *et al.* 1929, LADELL 1945, GERKING and ROBINSON 1946) or by repeated administration of drugs like metacholine (THAYSEN and SCHWARTZ 1955). In the present experiments, after sweating could no more be induced through repeated exposures to intense heat, exercise still was able to stimulate copious sweating in all subjects. This occurred, while the rectal temperature was on an average 0.7° C lower than during the preceding exposure to heat. Either the sweat glands rapidly recovered from the exhaustion, or the "fatigue" of the sweating mechanism was not at all due to the exhaustion of the glands.

The skin temperature is markedly different under the two conditions: in the "sauna", the mean skin temperature may rise to 42° C (PIIRONEN and ÄIKÄS 1960), while at exercise it obviously does not exceed body temperature. According to ROBINSON and GERKING (1947), the sweating mechanism is "fatigued" the sooner, the higher the skin temperature. With this observation, the present results are in an agreement.

The sodium and chloride concentration of thermal sweat is known to increase, as sweating becomes more profuse (KITTSTEINER 1911, 1913, DILL *et al.* 1938, BÖTTNER and SCHLEGEL 1940, KYUTOKU 1940, CHUN 1942, LOCKE *et al.* 1951). On the other hand, the sodium and chloride content of sweat is reported

to rise progressively, as "fatigue" develops during prolonged thermal sweating (ROBINSON and ROBINSON 1954). In the present series, however, the sodium and chloride concentration remained essentially unchanged or slightly decreased with repeated exposures. In successive samples of sweat taken during an exposure to heat in the "sauna", the sodium and chloride concentration do not change, whereas the potassium concentration decreases (AHLMAN et al. 1953). In this respect the situation resembles that due to repeated injections of metacholine: a "fatigue" of the sweating mechanism ensues, without any change of the sodium content (DOLE et al. 1951).

The sweat sodium and chloride concentration is reported also to increase with the skin temperature (KITTSTEINER 1913, LEHMANN and SZAKALL 1939, JOHNSON et al. 1944, LADELL 1945, ROBINSON et al. 1950, WEINER and VAN HEYNINGEN 1952). The tendency to a lower sodium concentration in the exercise tests — with lower skin temperature — is in accordance with this finding. However, when sweating has been induced on different days either by heat or exercise, no regular differences have been observed in the electrolyte composition (HASAN et al. 1954).

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INDEX

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